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Antiviral Responses in Mouse Embryonic Stem Cells: Differential Development of Cellular Mechanisms in Type I Interferon Production and Response

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ANTIVIRAL RESPONSES IN MOUSE EMBRYONIC STEM CELLS:
DIFFERENTIAL DEVELOPMENT OF CELLULAR MECHANISMS
IN TYPE I INTERFERON PRODUCTION AND RESPONSE

by

Ruoxing Wang

Abstract of a Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

August 2014

ABSTRACT

ANTIVIRAL RESPONSES IN MOUSE EMBRYONIC STEM CELLS:

DIFFERENTIAL DEVELOPMENT OF CELLULAR MECHANISMS

IN TYPE I IFN PRODUCTION AND RESPONSE

by Ruoxing Wang

August 2014

Embryonic stem cells (ESCs) have been recognized as a promising cell source for regenerative medicine. Intensive research over the past decade has led to the possibility that ESC-derived cells will be used for the treatment of human diseases. However, increasing evidence indicates that ESC-derived cells generated by the current differentiation methods are not fully functional. It is recently recognized that ESC-derived cells lack innate immunity to a wide range of infectious agents and inflammatory cytokines. When used in patients, ESC-derived cells would be placed in wounded sites that are exposed to various pathogens and inflammatory cytokines; therefore, their viability and functionality would be significantly compromised if the cells do not have competent immunity. The responses of mESCs to three types of live viruses, La Crosse virus, West Nile virus, and Sendai virus, were firstly investigated. The results demonstrated that mESCs were susceptible to the viral infections, but they were unable to express type I interferons (IFN α and IFN β). The failure of mESCs to express IFN α/β was further demonstrated with polyIC (polyinosinic-polycytidylic acid), a synthetic viral dsRNA analog that strongly induced IFN α/β in 10T1/2 cells. The author conclude that the mechanisms that mediate type I IFN expression are deficient in mESCs. It will be further demonstrated that single stranded RNA and

protein encoding mRNA induce strong IFN expression and cytotoxicity in fibroblasts and cancer epithelial cells, but none of these effects associated with antiviral responses was observed in ESCs. Therefore, ESCs are intrinsically deficient in antiviral responses; in particular, they do not have functional mechanisms to express type I IFN. Furthermore, the author found that mESCs can respond to type I IFNs and express IFN-stimulated genes as in differentiated fibroblasts. IFN β and IFN ω can protect mESCs from La Crosse virus -induced cell death and inhibit the replication of LACV and West Nile Virus. In summary, these findings illustrated that the cellular mechanisms for production of and response to type I IFN are not equally developed in mESCs; they are deficient in type I IFN expression but have functional mechanisms that respond to and mediate the antiviral effects of type I IFN.

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LIST OF ABBREVIATIONS

ESC: embryonic stem cell

ICM: inner cell mass

mESCs: mouse ESCs

hESCs: Human ESCs

LPS: lipopolysaccharide

IFN: interferon

PAMPs: pathogen associated molecular patterns

PRR: pattern recognition receptors

IRF: interferon-regulatory factor

ISGs: IFN-stimulated genes

TLRs: Toll like receptors

IL: Interleukin

PG: peptidoglycan

LTA: lipoteichoic acid

LM: Lipomannan

LAM: lipoarabinomannan

GPI: glycosylphosphatidylinositol

GIPLs: glycoinositolphospholipids

HSV: herpes simplex virus

MCMV: murine cytomegalovirus

dsRNA: Double-stranded RNA

poly I:C: polyinosinedeoxyctidylic acid

LCMV: lymphocytic choriomeningitis virus

VSV: vesicular stomatitis virus

RSV: respiratory syncytial virus

TIRAP: TIR-associated protein

MAL: MyD88- adaptor-like

TICAM1: TIR-domain-containing molecule 1

IRAK: IL-1R associated kinase

TNF: tumor necrosis factor

TRAF: TNFR-associated factor

NF- κ B: Nuclear factor κ B

NEMO: NF- κ B essential modulator

TAK: TGF- β -activated kinase

MAPK: Mitogen activated protein kinase

MKK: MAPK kinase

RIP1: receptor-interacting protein 1

TANK: TRAF family-member-associated NF- κ B activator

TBK: TANK binding kinase 1

NOD: nucleotide binding oligomerization domain

MDP: muramyl dipeptide

RIG-I: Retinoic acid inducible protein I

MDA5: Melanoma differentiation associated gene 5

RLR: RIG-I like receptor

IPS-1: IFN- β promoter stimulator 1

MAVS: mitochondrial antiviral signaling protein

VISA: virus-induced signaling adapter

TIM: TRAF-interacting motif

I κ B: protein kinase inhibitor of NF- κ B

FADD: Fas-associated death domain

RIP1: receptor interacting protein 1

TRADD: TNFR-associated death domain

PKR: double-stranded RNA dependent protein kinase

VV: vaccinia virus

eIF2: eukaryotic initiation factor 2

PACT: PKR-associated activator

MEF: Mouse embryonic fibroblasts

JAK: Janus family of tyrosine kinases

STAT: signal transducer and activator of transcription

ISGF-3: interferon stimulated gene factor 3

ISREs: interferon stimulated response elements

SOCS: suppressor of cytokine signaling

PIAS-1: protein inhibitor of activated stat-1

SHP-1: SH-2 containing phosphatase-1

OAS: 2',5'-oligoadenylate synthetase

ADAR: RNA-specific adenosine deaminase

ORF: open reading frame

ATF-4: activating transcription factor 4

Pre-RC: pre-replication complex

2,5-A: 2', 5'-oligoadenylate

dsRBD: dsRNA binding motifs

HDV: hepatic delta virus

ECS: editing complementary sequences

LACV: La Cross virus

HAEC: human aortic endothelial cells

hiPSCs: human induced pluripotency stem cells

WNV: West Nile virus

TB: toluidine blue

PI: propidium iodide

FITC: fluorescein isothiocyanate

ICAM: Inter-cellular adhesion molecule

VCAM: vascular cell adhesion molecule

ELISA: enzyme-linked immunosorbant assay

CDK: cyclin-dependent kinase

SeV: Sendai Virus

GFP: green fluorescence protein

RT: reverse transcription

PCR: polymerase chain reaction

LIF: leukemia inhibitory factor

RNAi: RNA interference

Ago2: Argonaute2

RISC: RNA induced suppressor complex

CHAPTER I

INTRODUCTION

Introduction of Embryonic Stem Cells and Related Research

Embryonic stem cells: isolation and their differentiation potential

Thirty years ago, Evans and Kaufman isolated and established the first embryonic stem cell (ESC) line from mouse embryos (Evans & Kaufman, 1981; Martin, 1981). As shown by Figure 1, ESCs are derived from pre-implantation stage embryos called blastocysts which consist of outer trophoblast cells and a group of inner undifferentiated cells referred to as the inner cell mass (ICM) (Wobus & Boheler, 2005). ESCs cultured in vitro can maintain properties of ICM cells and retain the potential to differentiate into all cell types of three germ layers in the embryo: ectoderm, mesoderm and endoderm. The fertilized egg and its early progeny (up to the 8-cell morula phase) have the ability to give rise to all cell types of the body as well as extra-embryonic tissues, a property known as totipotency, while cells from the ICM and ESCs are termed pluripotent, as they can only give rise to cell types of the embryo proper (Wobus & Boheler, 2005), not extra-embryonic tissues.

The differentiation potential of ESCs has been demonstrated both in vivo and in vitro. When introduced into blastocysts, exogenous ESCs could contribute to all cell lineages in three germ layers and the germ line in the resulting mouse chimeras (Bradley, Evans, Kaufman, & Robertson, 1984). The contribution to chimera formation is used as the gold standard for pluripotent cell lines. On the other hand, when induced in vitro,

ESCs can differentiate into various somatic cell types and cells in the germ line (Doetschman, Eistetter, Katz, Schmidt, & Kemler, 1985; Geijsen et al., 2004; Hubner et al., 2003; Toyooka, Tsunekawa, Akasu, & Noce, 2003; Wobus, Holzhausen, Jakel, & Schoneich, 1984). Once established, ESCs display an infinite capacity to proliferate and retain the differentiation potential to all cell lineages in vitro under proper conditions (Smith, 2001). The process through which ESCs reproduce themselves and maintain pluripotency is termed as self-renewal. This feature makes ESCs an ideal unlimited source for regenerative medicine and cell therapy.

Aside from potential medical applications of ESCs, these cells also have great value for studying gene functions and developmental biology. Several powerful technologies of gene manipulation have been successfully applied in ESCs, including overexpressing genes through random transgenesis, gene targeting to knock out genes or replace wild-type with mutant genes, and conditional knock-out and knock-in techniques (Gossler, Joyner, Rossant, & Skarnes, 1989; Lewandoski, 2001; Nagy, 2000; Thomas & Capecchi, 1987; Thompson, Clarke, Pow, Hooper, & Melton, 1989). Based on gene targeting, transgenic animal models can be established through re-introducing manipulated ESCs back into blastocysts to get heterozygous offspring with one mutated allele, followed by mating between heterozygous animals to obtain homozygous mutant offspring. These models have revealed functions of a great number of genes throughout the developmental process. The ESCs isolated from embryos of homozygous mutant animals can be studied in vitro even when in vivo studies are impossible because of

embryonic lethality. Targeting genes in chromosome pairs sequentially in ESCs is much less labor intensive and has proved indispensable in elucidating gene function. One example is the characterization of the Nanog gene in ESCs (Mitsui et al., 2003).

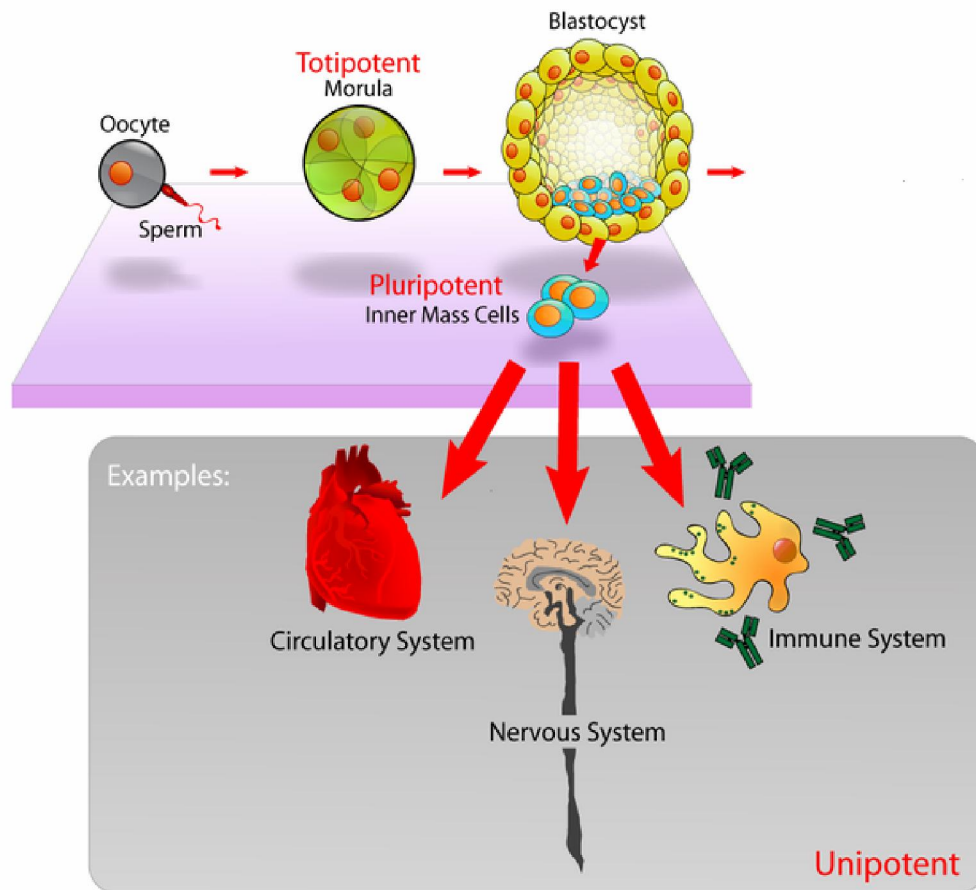


Figure 1. Derivation and differentiation potential of embryonic stem cells (Jones, 2006).

ESC based cell therapy and regenerative medicine: promises and challenges

ESCs have unlimited capacity for self-renewal and can be induced to differentiate into various cell lineages. These properties make them a promising source for cell-based therapy. The recent extensive research has led to the development of methods for ESC differentiation to different cell types. While these advances have

proven the principles of ESC-based cell therapy, it is also increasingly clear that generating clinically usable cells is a task that faces many biological and technical challenges. No ESC-derived cell preparations have so far been approved for medical application. Current knowledge about the basic physiology of ESCs is limited, and there are many safety issues that have yet to be resolved.

Mouse ESCs proliferate rapidly with truncated cell cycle times of 10-15 h (White & Dalton, 2005). During *in vitro* culture, ESCs display unlimited proliferative capacity (Suda, Suzuki, Ikawa, & Aizawa, 1987), clonal propagation, anchorage independence, and a lack of contact inhibition (Burdon, Chambers, Stracey, Niwa, & Smith, 1999). These features are shared by many malignant cells. It is well recognized that if ESCs are not fully differentiated and transplanted to the patient, they can grow into tumor-like structures known as teratomas (Stevens, 1983; Wobus et al., 1984). However, most current differentiation protocols fail to eliminate all undifferentiated cells. This phenomenon is proved by the fact that in animal models, transplantation of ESC-derived differentiated cells still results in teratoma formation, presumably by undifferentiated ESCs (Blyszczuk et al., 2003; Kim et al., 2002). Many other issues have not been fully recognized. Currently, ESC-derived cells are mainly identified by cell-specific markers, which may reveal little of their functionality.

Until now, most knowledge about ESCs was derived from mouse ESCs (mESCs). Human ESCs (hESCs) isolated later have been shown to share some fundamental characteristics of murine cell lines such as telomerase activity, Oct-4

expression, and differentiation potential to three germ layers (Richards, Fong, Chan, Wong, & Bongso, 2002; Thomson et al., 1998). Also, hESCs can maintain proliferative potential and a normal karyotype for long periods of culture (Amit et al., 2000).

However, hESCs differ from mESCs in cell cycle time (30-35 h vs 10-15 h) and culture requirements (Amit et al., 2000; Reubinoff, Pera, Fong, Trounson, & Bongso, 2000; Thomson et al., 1998). Moreover, activation of the JAK/STAT3 pathway by LIF (which shares a similar signaling paradigm with IFN) is essential for the maintenance of self-renewal and pluripotency in mESCs but is not required for hESCs (Daheron et al., 2004; Humphrey et al., 2004; Matsuda et al., 1999). Whether the differences between human and mouse ESCs are caused by a difference between species or that in developing phases is still not clear, and the mechanisms behind remains elusive.

During long term in vitro culture, there is no guarantee of the genetic stability of ESCs. Somatic cells accumulate approximately one mutation through each turn of the cell cycle (Kunkel & Bebenek, 2000). Aside from gene mutation, in vitro environments can also change epigenetic modifications which play important roles in the developmental potential of ESCs. Stresses and potential contamination also need to be taken into consideration. A thorough understanding of mechanisms governing such ESC behaviors as self-renewal, proliferation, and differentiation is needed to improve the existing culture and differentiation methods.

Impacts of ESC research on basic research, cell biology, and development biology

Organismal development from the zygote to an adult is the most complex biological process and is far from being elucidated. Differentiation from totipotent to various somatic cells is the basis of development. During differentiation, cells undergo global epigenetic changes resulting in new expression patterns of genes with special functions. The study of how epigenetic states and expression of numerous genes are regulated in early embryonic cells has greatly benefited from ESC research. ESCs, the in vitro counterpart of pluripotent cells in the early embryos, have proved a valuable model to investigate how “stem” and “differentiation” genes are regulated during differentiation (Bernstein et al., 2006; Guenther et al., 2010; Guenther, Levine, Boyer, Jaenisch, & Young, 2007; Lee et al., 2006). These data obtained from such investigations are fundamental to elucidate the mechanisms involved in cell differentiation, tissue formation, and organismal development.

Studies of ESCs showed that they share many features with cancer cells such as infinite proliferation ability, rapid cell cycle progression, deficient cell cycle checkpoints, and anchorage-independent growth (White & Dalton, 2005). However, while keeping such tumor characteristics, ESCs have much a lower spontaneous mutation rate and a higher tolerance and resistance to exogenous stresses (Hong, Cervantes, Tichy, Tischfield, & Stambrook, 2007; Maynard et al., 2008; Saretzki et al., 2008). The similarities between cancer cells and ESCs in fact brought about the “cancer stem cell” hypothesis, which states that not all cancer cells are the same and a particular

population of cancer cells has the unique properties like ESCs that give rise to all cell types in a particular cancer type with invasive capacity. Therefore, the study of ESCs has provided a model to help understand cancer biology and disease modeling. Special regulation of proliferation and maintenance of genetic integrity makes ESCs a valuable model when studying mechanisms governing cell propagation, response to stress and genetic stability, and how these mechanisms are altered in cancer cells (Lin, Donahue, & Ruley, 2006; Tichy & Stambrook, 2008).

Innate Immunity and Type I IFN Signaling

Many types of adult somatic cells in organisms are exposed to external environments containing various pathogenic microorganisms. To protect the whole organism from infection, a sophisticated immune system comprised of immune cells that specifically recognize pathogens and non-specific innate immunity has been established in vertebrates. After exposure to components of pathogens like bacterial lipopolysaccharide (LPS) or viral RNA, several signaling pathways are activated, leading to changes in metabolism and gene expression in infected cells. Consequential immune responses are then activated to limit and remove pathogenic infection. So, the overall immune response begins with innate immune system activation in infected cells and continues with innate or specific immunity of immune cells.

While most differentiated somatic cells have already established innate immunity, understanding how ESCs respond to pathogens is not only interesting in stem cell biology but also valuable for their application in cell therapy. Will pathogen exposure and

the responses of ESCs affect their stem cell state? When and how is innate immunity established during differentiation? Could innate immunity be normally established during differentiation of cultured ESCs? To answer these questions, the responses of ESCs to pathogens should be thoroughly investigated.

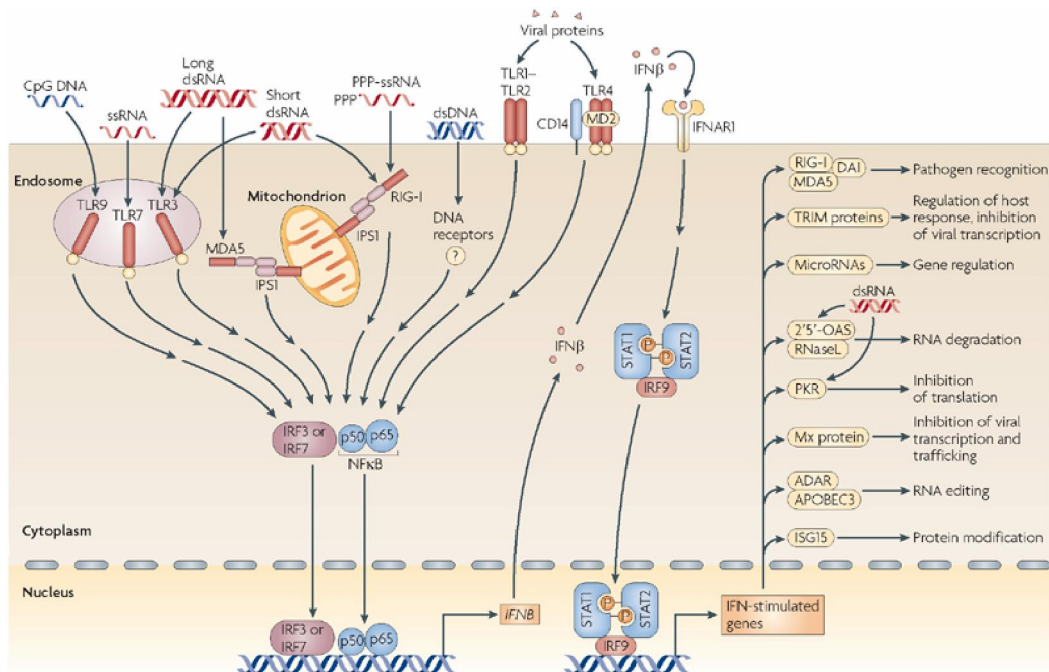


Figure 2. Type I IFN in anti-viral innate immunity. Pathogen recognition receptors (PRRs) detecting viral components transduce signaling to activate transcription factors: interferon-regulatory factor 3 (IRF3), interferon-regulatory factor 7 (IRF7) and nuclear factor- κ B (NF κ B), which induce expression of type I IFN. After being synthesized and secreted, type I IFNs bind their receptors, activate downstream signaling, mediate expression of IFN-stimulated genes (ISGs) and finally initiate an anti-viral effector program (Bowie & Unterholzner, 2008).

As shown in Figure 2, various receptors recognize invading components and transduce signals to trigger expression of type I IFNs (IFN α/β) and inflammatory cytokines. Then, these cytokines induce further cellular events in infected and neighboring cells and recruit immune cells. Detailed information about the type I IFN signaling will be introduced in corresponding sections.

Innate Immunity in ESCs

Most studies of innate immunity are done in differentiated somatic cells, especially in cells with immune-related roles. It is not clear whether innate immunity exists in early embryos, or how and when innate immunity is established during development.

Since ESCs are a promise source for regenerative medicine, their differentiated progeny should be fully functional to meet medical and physiological demands. Innate immunity is a pivotal function of hematopoietic cells, vascular cells, and cells directly exposed to the environment. Recent studies have shown that hESCs, unlike somatic cells, exhibit an attenuated response to dsRNA (Chen, Yang, & Carmichael, 2010). Expression of immune sensors is low or undetectable in hESCs, and induction of cytokines is much weaker than in somatic cells (Chen et al., 2010). Moreover, cellular localization of dsRNA in hESCs is different from in differentiated cells (Chen et al., 2010). hESCs were also found to express few TLR members and do not respond to TLR ligands. What is more interesting is that even hESC-derived endothelial cells and cardiomyocytes after 4 months of differentiation respond poorly to pathogen associated molecular patterns, (PAMP) compared with primary human aortic endothelial cells (HAEC) (Foldes et al., 2010).

It is also interesting to note that a recent study suggested that hESCs and human induced pluripotency stem cells (hiPSCs) have limited or no capacity to respond to IFN (Hong & Carmichael, 2013).

In summary, how ESCs respond to PAMPs is still elusive, and data about the developing status of innate immune system in ESCs remain limited and controversial.

CHAPTER II

OBJECTIVE AND SIGNIFICANCE

ESCs have unlimited capacity for self-renewal and can be induced to differentiate into any cell lineage (Wobus & Boheler, 2005). These properties make them a promising source for cell-based regenerative medicine. The extensive research over the past decade has led to the development of methods for ESC differentiation to various cell types. These advances have proven the principles of ESC-based cell therapy. However, it is also increasingly clear that generating clinically usable cells is a task filled with many biological and technical challenges. For example, while it is well-recognized that if ESCs are not fully differentiated and transplanted to the patient, they can grow into tumor-like structures called teratomas; some other potential problems have not yet been fully recognized (Stevens, 1983). Currently, ESC derived cells are mainly identified by cell-specific markers which may reveal little about their functions. An important issue is innate immunity, which is not easily recognized in normal cells without challenge from infectious agents. Only a few studies, including our own, have investigated the innate immunity and inflammation responses of ESC-derived cells (Wang et al., 2013). Such studies have demonstrated that endothelial cells and smooth muscle cells derived from both human and mouse ESCs have limited ability to respond to viral related infectious agents, LPS, and inflammatory cytokines (Foldes et al., 2010; Glaser et al., 2011). The deficiency in the immune and inflammatory responses is likely found in most, if not all, types of ESC-derived cells as indicated by my recent data. Therefore, the objective of my

work is to investigate the innate immunity of undifferentiated mESCs. The author hypothesize that undifferentiated ESCs do not have fully functional innate immunity, which is supposed to be established in somatic cells.

While ESC research has led to a great expectation that ESC-derived cells could soon be used for regenerative medicine, in reality these cells will not become clinically beneficial if they cannot perform the essential functions of their in vivo counterparts. The molecular mechanisms regulating proliferation, pluripotency, and self-renewal have been the center of ESCs research for the past decade. However, developing strategies for functional ESC-derived cells usable for clinical application may mark the beginning of a new era of ESC research. Innate immunity is an important function of normal in vivo differentiated somatic cells; therefore, studying its development may contribute to the design of differentiation strategies that generate cell lineages with active innate immunity. Moreover, understanding the development of innate immunity during differentiation will be not only instructive for the derivation of many cell types from ESCs with competent immunity, but also fundamental to understand the basic biology of ESCs and pathogen-host interaction.

CHAPTER III

MOUSE EMBRYONIC STEM CELLS ARE DEFICIENT IN TYPE I INTERFERON
EXPRESSION IN RESPONSE TO VIRAL INFECTIONS
AND DOUBLE-STRANDED RNA

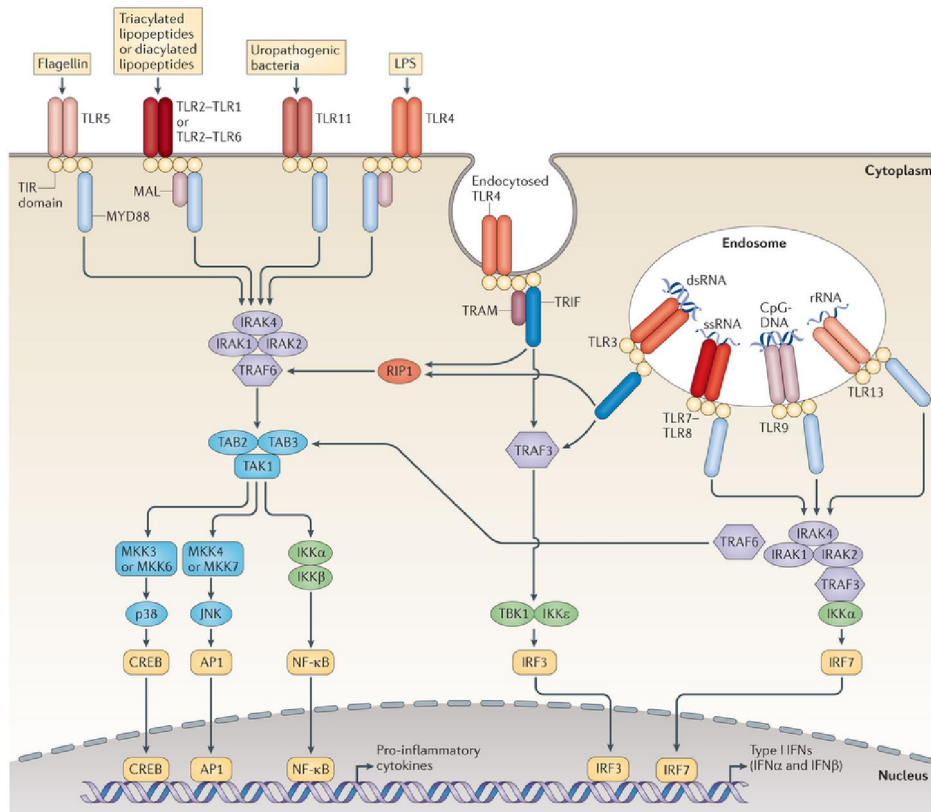
Introduction

The anti-viral immune response, which is centered on type I IFN production and response, starts with the recognition of specific pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRR), and induction of type I IFNs is the central part of innate immunity. There are various PRRs and pathways leading to production of type I IFNs.

Toll-like receptors

Toll-like receptors (TLRs) are a family of conserved transmembrane proteins with critical roles in innate immunity (Akira, Uematsu, & Takeuchi, 2006). TLRs expressed at the cellular membrane or membranes of the intracellular organelles and endocytic vesicles recognize various microbial molecular structures such as LPS, lipopeptides, viral nucleic acids (Akira et al., 2006). TLRs are type I integral transmembrane glycoproteins containing extra-membrane domains and an intra-membrane domain homologous to the intracellular domain of interleukin 1 receptor (IL-1R) (Bowie & O'Neill, 2000). Different TLR members have different cellular locations. While TLR1, 2, 4, 5, and 6 are found on the cell membrane, TLR3, 7, 8, and 9 are expressed almost exclusively on the membranes of endosomes (Akira et al., 2006).

TLRs with various primary sequences recognize diverse microbial targets and can be divided into several subclasses.



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Figure 3. Toll-like receptor signaling. TLRs localized on the cellular membrane and endosomes recognize various pathogen components, form dimers and finally activate two important families of transcription factors: nuclear factor- κ B (NF- κ B) and the interferon-regulatory factors (IRFs), leading to induction of Type I IFN and pro-inflammatory cytokines (Bowie & Unterholzner, 2008).

LPS, a best studied bacterial endotoxin, is recognized by TLR4, leading to their oligomerization (Politorak et al., 1998; Shimazu et al., 1999). Unlike Gram negative bacteria, Gram positive bacteria do not synthesize LPS but still can be recognized by innate immunity. TLR2, in some cases together with TLR1 and TLR6, recognizes bacterial components such as lipoproteins, peptidoglycan (PG), and lipoteichoic acid (LTA) (Alexopoulou et al., 2002; Ozinsky et al., 2000; Takeuchi et al., 2001; Takeuchi et

al., 2002). Flagellin, the major component of flagella, is also a potent activator of innate immunity. TLR5 which is responsible for this process recognizes a conserved and constant domain of flagellin (Hayashi et al., 2001). TLR9 recognizes bacterial genome DNA and triggers the innate immune response (Hemmi et al., 2000).

Mycobacterial cell wall components, lipomannan (LM), and lipoarabinomannan (LAM) have been shown to activate TLR2 and TLR4, and LAM captured by a phosphoinositide residue (PILAM) is a potent stimulator of TLR2 (Akira et al., 2006; Gilleron, Quesniaux, & Puzo, 2003). In addition, TLR2 together with TLR1 can recognize cell-wall associated lipoprotein in *M. tuberculosis* (Thoma-Uszynski et al., 2001).

Several cell wall components are recognized by TLR2 and TLR4. TLR4 knockout mice showed increased susceptibility to disseminated *Candida* infection, while TLR2 null mice exhibited increased resistance (Netea, Van der Graaf, Van der Meer, & Kullberg, 2004). Although TLR-induced signals activate Th1-directed responses that are critical for protection against fungi, TLR2 activation is less inflammatory and leads to Th2 response through inducing IL-10 expression (Agrawal et al., 2003). A trans-membrane protein Dectin-1 collaborates with TLR2 to elicit a strong inflammatory response through recruitment of the protein tyrosine kinase Syk in response to yeast (Gantner, Simmons, Canavera, Akira, & Underhill, 2003; Rogers et al., 2005; Underhill, Rossmagle, Lowell, & Simmons, 2005).

Components of protozoan parasites are also sensed by TLRs.

Trypanosoma-derived molecules, glycosylphosphatidylinositol mucin (tGPI-mucin), glycoinositolphospholipids (GIPLs), and genomic DNA could activate TLR2, 4, 9 respectively (Gazzinelli, Ropert, & Campos, 2004). TLR11 has been reported to recognize a profilin-like molecule in the extract from *T. gondii* tachyzoites (Yarovinsky et al., 2005). In addition, some non-DNA molecules have been shown to activate TLR9 (Coban et al., 2005; Pichyangkul et al., 2004).

Besides bacteria and protozoans, viruses also activate innate immunity through multiple TLRs and other receptors.

Aside from bacterial DNA, TLR9 also recognizes viral DNAs. DNA viruses as herpes simplex virus 1 (HSV-1), HSV-2, and murine cytomegalovirus (MCMV) with genomes rich in CpG-motifs induce expression of IFN and inflammatory cytokines through binding TLR9 (Hochrein et al., 2004; Krug et al., 2004a; Krug et al., 2004b; Lund, Sato, Akira, Medzhitov, & Iwasaki, 2003). Mouse TLR7 and human TLR8 recognize uridine-rich or uridine/guanosine-rich single-stranded RNA of both viral and host origins (Diebold, Kaisho, Hemmi, Akira, & Reis e Sousa, 2004; Heil et al., 2004; Hemmi et al., 2002). Double-stranded RNA (dsRNA) and its analog, polyinosinic-polycytidylic acid (poly I:C), are potent activators of TLR3 and inducers of type I IFN (Alexopoulou, Holt, Medzhitov, & Flavell, 2001). However, several lines of evidence indicate that TLR3 is not required for initial, cell-autonomous recognition of viral infection, and the first wave of type I IFN induction (Lopez et al., 2004). TLR3

knockout mice do not exhibit increased susceptibility to MCMV, lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), and reovirus infection (Edelmann et al., 2004). These findings may indicate the existence of other components for dsRNA recognition. Components of viral envelope also have been shown to activate TLRs such as TLR2 and TLR4. For instance, TLR4 can recognize the fusion protein from respiratory syncytial virus (RSV) causing infiltrating inflammation (Kurt-Jones et al., 2000). TLR2 can also be activated by viral components as measles virus (MV) hemagglutinin, human CMV, and HSV-1 (Bieback et al., 2002; Compton et al., 2003).

After ligand binding, TLRs dimerize and undergo conformational changes, leading to the recruitment of their adaptors, including MyD88, TIR-associated protein (TIRAP)/MyD88- adaptor-like (MAL), TIR-domain-containing adaptor protein- inducing IFN- β (TRIF)/TIR-domain-containing molecule 1 (TICAM1), and TRIF-related adaptor molecule (TRAM) (Akira et al., 2006; Oshiumi, Matsumoto, Funami, Akazawa, & Seya, 2003; Yamamoto et al., 2002b).

Upon activation of TLRs, MyD88 associates with the intracellular domain of TLRs and recruits IL-1R associated kinase 4 (IRAK4) and IRAK1 (Akira et al., 2006). TLR2 and TLR4 require another adaptor, TIRAP/Mal to recruit MyD88 (Fitzgerald et al., 2001; Horng, Barton, Flavell, & Medzhitov, 2002; Horng, Barton, & Medzhitov, 2001; Yamamoto et al., 2002a). IRAK1 then is phosphorylated by IRAK4 and associates with TNFR-associated factor 6 (TRAF6), an E3 ubiquitin ligase (Li, Strelow, Fontana, & Wesche, 2002). TRAF6 then promotes the formation of K63-linked polyubiquitin chain

on TRAF6 itself and on IKK-g/NF- κ B essential modulator (NEMO) (Deng et al., 2000). At the same time, TRAF6 also recruits a complex composed of TGF- β -activated kinase 1 (TAK1) and the TAK1 binding proteins TAB1, TAB2, and TAB3 (Wang et al., 2001). This complex phosphorylates IKK- β and MAPK kinase 6 (MKK6) to modulate the activity of NF- κ B and MAPK pathway (Wang et al., 2001).

In addition, after TLR activation transcription factor IRF-5 translocates to the nucleus and binds promoter regions of cytokine genes, leading to their up-regulation (Takaoka et al., 2005). I κ B ζ is rapidly induced by TLR binding to ligands and associates with NF- κ B p50 to stimulate expression of inflammatory cytokines such as IL-6 and IL-12 (Yamamoto et al., 2004).

Another set of important downstream genes induced by TLRs is type I IFN. IFN is induced by TLR3, 4, 7 and 9 ligands (Akira et al., 2006). One way to activate IFN transcription is through activation of NF- κ B pathway via MyD88. The other pathway, which is MyD88-independent, can be triggered by TLR3 and TLR4 (Kaisho, Takeuchi, Kawai, Hoshino, & Akira, 2001). This MyD88 independent pathway is mediated by another TRIF and, in the case of TLR4, assisted by TRAM, which bridges TLR4 and TRIF (Fitzgerald et al., 2003b; Hoebe et al., 2003; Yamamoto et al., 2003a; Yamamoto et al., 2003b). Upon TLR activation, TRIF interacts with receptor-interacting protein 1 (RIP1) and, on the other hand, activates TRAF family-member-associated NF- κ B activator (TANK) binding kinase 1 (TBK1; also known as NAK or T2K) via TRAF3 (Hacker et al., 2006; Meylan et al., 2004; Oganessian et al., 2006). TBK1 consists of a

family of inducible I κ B kinase (IKK- ϵ) which directly phosphorylates IRF-3 and IRF-7 (Fitzgerald et al., 2003a; Sharma, 2003). IRF-3 and IRF-7 are essential for type I IFN induction as evidenced by the abrogation of virus-induced IFN production in IRF3/7 knockout cells (Honda et al., 2005).

In addition, IRF7, in some circumstances, also contributes to IFN induction in a MyD88-dependent manner with IRAK-1, IRAK-4, and TRAF-6 (Honda et al., 2004; Kawai et al., 2004).

RIG-I like receptor (RLR) family

TLRs are able to recognize dsRNA and stimulate type I IFN expression; however, most virus-infected cells produce IFN in a TLR-independent manner (Akira et al., 2006). Retinoic acid inducible protein I (RIG-I) containing CARD domains and a DExD/H box helicase domain serves as a cytoplasmic dsRNA sensor (Yoneyama et al., 2004). Melanoma differentiation associated gene 5 (MDA5), which is homologous to RIG-I, has also been implicated in recognizing dsRNA (Andrejeva et al., 2004; Kang et al., 2002). These two proteins belong to the RIG-I like receptor (RLR) family. Another member of this family is LGP2, which has a homologous helicase domain but lacks CARD (Rothenfusser et al., 2005; Yoneyama et al., 2005). LGP2 is considered to be a negative regulator of RIG-I and MDA5.

Although similar in structure, RIG-I and MDA5 have been shown to play different roles in recognizing RNA viruses (Kato et al., 2005). Some viruses activate only one of them while some are recognized by both (Eisenaher & Krug, 2012).

Besides dsRNA, RIG-I can also be activated by exogenous and microbial DNA.

This process is mediated by RNA polymerase III that transcribes the DNA to RNA

recognizable for RIG-I (Ablasser et al., 2009; Chiu, Macmillan, & Chen, 2009). These

DNAs are transcribed in great amount by host RNA pol III to activate RIG-I.

Recognition of ssRNA by RIG-I depends on the 5' tri-phosphate motif in ssRNA

(Hornung et al., 2006; Pichlmair et al., 2006). This recognition mechanism may provide a

method to distinguish host RNAs from viral RNAs (Rmos & Gale, 2011).

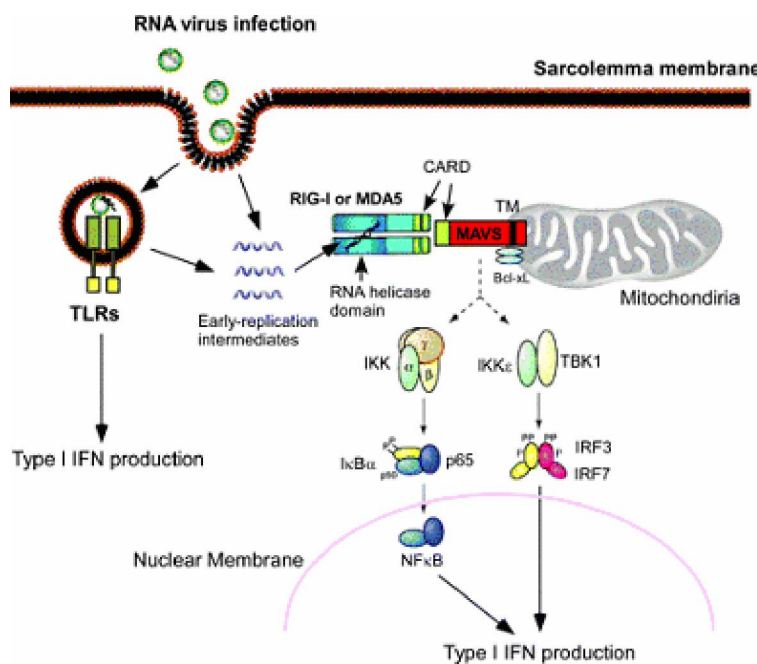


Figure 4. Signaling transduction of RLRs. RLRs bind MAVS on the surface of mitochondria and induces activation of IKK and TBK1, stimulating nuclear factor (NF)- κ B and interferon (IFN) regulatory factor (IRF)–dependent pathways respectively. (Yajima & Knowlton, 2009)

Shortening synthesized dsRNA polyIC shifts its recognition by MDA5 to RIG-I,

suggesting that MDA5 may prefer dsRNA with longer base pairs (Kato et al., 2008).

Recent data indicate that higher order dsRNA “web” structure is responsible for MDA5

activation rather than simply long length (Kato et al., 2008; Pichlmair et al., 2009).

As shown in Figure 4, upon activation and dimerization of RLRs, adapter protein IFN- β promoter stimulator 1 (IPS-1) with CARDs binds RLRs and transduces signals (Kawai et al., 2005). This protein is independently found and named mitochondrial antiviral signaling protein (MAVS), virus-induced signaling adapter (VISA), and CARD adapter inducing IFN- β (CARDIF) (Meylan et al., 2005; Seth, Sun, Ea, & Chen, 2005; Xu et al., 2005). Overexpression of IPS-1 enhances RLR mediated immune response, while IFN induction by RIG-I- or MDA5- recognized viruses is impaired in IPS-1 null mice, indicating its essential roles (Kumar et al., 2006; Sun et al., 2006). In addition to receptor-adaptor interaction, the function of IPS-1 relies on its localization at the outer membrane of mitochondria (Seth et al., 2005).

In addition to mitochondria localization, IPS-1 has also been found in peroxisomes (Dixit et al., 2010). This population mainly induces rapid IRF1/3 dependent expression of IFN stimulated genes (ISGs), leading to an early but transient anti-viral response independent of type I IFN (Dixit et al., 2010).

Upon CARD-CARD interaction between receptors and adaptors, tumor necrosis factor receptor-associated factor (TRAF) family members are required. Interaction of IPS-1 with TRAF3 through its TRAF-interacting motif (TIM) is essential for IPS-1 dependent IFN induction (Saha et al., 2006). On the other hand, TRAF2 and TRAF6 interact with IPS-1 to induce NF- κ B activation (Xu et al., 2005). The downstream of IPS-1, TRAFs transmit signals to the protein kinase inhibitor of NF- κ B (I κ B) kinase complex composed of NEMO(IKK γ):IKK α :IKK β . Then, the kinase complex

phosphorylates I κ B, promoting its degradation and NF- κ B nuclear translocation and activation (Karin & Ben-Neriah, 2000; Xu et al., 2005).

As shown in Figure 4, IPS-1 activates IRF3 and IRF7 in parallel through two IKK related kinases: TBK1 and IKKi (Honda & Taniguchi, 2006). Phosphorylated IRF3 and IRF7 form homo-/heterodimers that translocate to nucleus and induce type I IFN expression (Honda & Taniguchi, 2006; Honda et al., 2005). Furthermore, NEMO, with essential functions in RLR-induced NF- κ B activation, also contributes to TBK/IKKi-induced IRF activation (Zhao et al., 2007).

Moreover, Fas-associated death domain (FADD) and receptor interacting protein 1 (RIP1) associate with IPS-1 and recruit caspase 8 and 10, activating downstream NF- κ B signaling (Balachandran, Thomas, & Barber, 2004; Takahashi et al., 2006). The DD-containing protein, TNFR-associated death domain (TRADD), associates with IPS-1, TRAF3, TANK as well as FADD and RIP1 to activate both IRF3 and NF- κ B pathways (Michallet et al., 2008).

Double-stranded RNA dependent protein kinase (PKR)

Several early observations led to the discovery of double-stranded RNA dependent protein kinase (PKR). IFN-treated cells infected with vaccinia virus (VV), which produces dsRNA, have a translational block of viral and cellular mRNAs (Colby & Duesberg, 1969; Friedman et al., 1972; Metz & Esteban, 1972). Also, dsRNA were known to inhibit protein synthesis in cells and cell-free systems (Cordell-Stewart & Taylor, 1973; Ehrenfeld & Hunt, 1971). Later, it was found that a dsRNA-dependent

kinase could inhibit the translation of mRNA by phosphorylating the alpha subunit of eukaryotic initiation factor 2 (eIF2 α) (Farrell, Balkow, Hunt, Jackson, & Trachsel, 1977). This kinase was found to phosphorylate not only eIF2 α but also the kinase itself (Krust, Galabru, & Hovanessian, 1984; Laurent, Krust, Galabru, Svab, & Hovanessian, 1985). Finally, the kinase was cloned and termed PKR (Clemens et al., 1993; Meurs et al., 1990).

PKR is a serine/threonine kinase having the kinase domain shared with other eIF2 α kinases and two dsRNA binding domains regulating its activity. In non-stressed cells, PKR is kept in a monomeric inactive state due to the inhibitory effects of the dsRNA binding domains. Double-stranded RNA, as the main stimulator of PKR, binds the dsRNA binding motifs, resulting in PKR activation and autophosphorylation (Clemens, 1997). PKR autophosphorylation represents its activation and leads to phosphorylation of eIF2 α (Galabru & Hovanessian, 1987; Hovanessian, 1989). To effectively activate PKR, dsRNA should be longer than 30bp, correlating with the structure of dsRNA binding domains of PKR (Manche, Green, Schmedt, & Mathews, 1992). In addition to dsRNA, PKR can be activated by polyanions as heparin, dextran sulfate, chondroitin sulfate, and poly-L-glutamine (Hovanessian & Galabru, 1987).

After binding dsRNA, PKR undergoes a series of conformational changes and homodimerization, which are important for its activation and autophosphorylation (Dey et al., 2005). Autophosphorylation on several residues as Thr446 and Thr451 further stabilizes dimer formation (Dey et al., 2005; Taylor et al., 1996; Zhang et al., 2001).

Double-stranded RNA has long been shown to activate NF- κ B activity (Visvanathan & Goodbourn, 1989). Down-regulation of PKR expression impairs NF- κ B activation in response to dsRNA (Maran et al., 1994). PKR knockout MEF exhibits impaired activation of NF- κ B and the induction of IFN under polyIC treatment (Yang et al., 1995). IKK γ /NEMO, a component in IKK complex that promotes degradation of I κ B, is essential for PKR-induced activation of NF- κ B, implying the involvement of IKK in the PKR-NF- κ B pathway (Yamaoka et al., 1998). The involvement of IKK complex in PKR induced NF- κ B activation in response to viral or synthesized dsRNA has been confirmed by several studies using the MEF lacking function of certain IKK proteins (Bonnet, Weil, Dam, Hovanessian, & Meurs, 2000; Chu et al., 1999; Gil, Alcamí, & Esteban, 2000; Zamanian-Daryoush, Mogensen, DiDonato, & Williams, 2000). These studies also revealed physical interaction between PKR and IKK complex (Bonnet et al., 2000; Gil et al., 2000; Zamanian-Daryoush et al., 2000).

TNF receptor associated factors (TRAF) are key signal transducers in many pathways other than TNF receptor signaling (Chung, Park, Ye, & Wu, 2002). PKR possesses putative TRAF-interacting motifs, and interaction between them has been confirmed (Gil et al., 2004). TRAF2 and TRAF5 were found to act downstream of PKR and transduce signals toward NF- κ B activation (Gil et al., 2004). In addition, TRAF3 was found to be essential for PKR-induced production of type I IFN (Oganesyan et al., 2006).

PKR is involved in many kinds of signaling pathways downstream of various growth factors and cytokines. IFN $\alpha/\beta/\gamma$ are transcriptional activators of PKR, while the

transcription of IFN γ is self-regulated by a pseudo-knot and followed by PKR activation (Ben-Asouli, Banai, Pel-Or, Shir, & Kaempfer, 2002). PKR also mediates IFN- γ triggered activation of NF- κ B and c-myc expression in a STAT1 independent manner (Deb, Haque, Mogensen, Silverman, & Williams, 2001a; Ramana et al., 2000). In addition, in synergy with TNF- α , PKR activates NF- κ B via affecting IKB β stability downstream of IFN γ (Cheshire, Williams, & Baldwin, 1999). Inhibiting or down-regulating PKR results in slight but sustained and reproducible defects in TNF- α induced NF- κ B activation (Kumar et al., 1997; Maran et al., 1994).

Methods

mESC Culture

D3 cells, a commonly used mESC line in the literature, were obtained from ATCC. They were used for the majority of the experiments in this study. The key experiments were repeated in DBA252 mESCs previously characterized (Guo, Ye, & Huang, 2007). Both cell lines were maintained in the standard mESC medium (Guo et al., 2007). Raw 264.7 (Raw) and 10T1/2 cells were cultured in DMEM that contains 10% fetal calf serum and 100 units/ml penicillin and 100 μ g/ml streptomycin. All cells were maintained at 37 °C in a humidified incubator with 5% CO₂.

Cell treatment

mESCs and 10T1/2 were plated at ~40% and ~70% confluence, respectively and cultured for 24 h before the experiments. For viral infection, viral stocks were added to the cell culture at the concentrations as specified in individual experiments. PolyIC (Sigma)

was either directly added to the cell culture or was transfected into the cells with DharmaFECT reagent (Thermo Scientific). For polyIC transfection experiments, control cells were transfected with DharmaFECT reagent only. The culture medium and treated cells were collected at different time periods and used for various analyses.

Preparation of viral stocks

La Crosse virus (LACV, stain name) and West Nile virus (WNV, stain 2471) were propagated in vero cells (African green monkey kidney cell line, ATCC). Titers of virus stocks were determined by plaque assay as previously described (22). Sendai virus (SeV, Cantell strain) stock was purchased from Charles River laboratory.

Real-time quantitative polymerase chain reaction (RT-qPCR)

RNA Extraction: Total RNA was extracted using Tri-reagent (Sigma). Medium was aspirated from the culture dish, and Tri-reagent was added followed by 3 minutes of shaking. The sample was transferred to a 1.5 mL microcentrifuge tube and stored at -70°C. Chloroform (0.2 mL per mL of Tri-reagent) was added. Samples were then vortexed for 15 seconds and incubated at room temperature for 10 minutes. Next, samples were centrifuged at 10000 rpm for 10 minutes at 4°C. The aqueous supernatant containing RNA was transferred to a new 1.5 mL microcentrifuge tube, and an equal volume of isopropyl alcohol was added. After a 10 minute incubation at room temperature, samples were centrifuged at 12000 rpm for 15 minutes at 4°C to precipitate a pellet of RNA. The supernatant was aspirated, and samples were washed with 0.7 mL cold 75% ethanol, vortexed, and placed in -20°C for at least one hour. Samples were then

centrifuged at 10000 rpm for 10 minutes at 4°C, ethanol was removed, and RNA pellets were allowed to dry for 5 minutes on ice. Finally, RNA was dissolved in 20 uL DEPC-water, RNA concentration was measured by a Spectronic Genesys 10 Bio spectrophotometer, and RNA integrity was checked by agarose gel electrophoresis.

Reverse Transcription: For each sample, 1 µg of RNA was mixed with dNTPs and a random primer, mixed, and held at 70°C in a thermal cycler for 5 minutes to melt secondary structure within the RNA template. Then a 5x buffer (Fisher), M-MLV reverse transcriptase (Fisher), and RNase inhibitor (Fisher) were added for a total volume of 20 µL per reaction, and samples were held at 42°C for a 1 hour extension step, followed by 10 minutes at 95°C to inactivate the reverse transcriptase enzyme. Resulting cDNA was diluted in 200 µL DEPC-water.

Real-time quantitative polymerase chain reaction (RT-qPCR): Total RNA was extracted using Tri-reagent (Sigma). cDNA was prepared by M-MLV reverse transcriptase (Sigma). RT-qPCR was performed using SYBR green ready mix on a MX3000PTM RT-PCR system (Stratagene), as previously reported (Guo et al., 2007). The mRNA level from RT-qPCR was calculated using the comparative Ct method (Pfaffl, 2001). β -actin mRNA was used as a calibrator for the calculation of relative mRNA of the tested genes. The sequences of the primer sets are listed in Table 1.

Table 1

Primer sequences for RT-qPCR

Gene	Sequence (forward)	Sequence (reverse)
β -actin	CATGTACGTAGCCATCCAGGC	CTCTTTGATGTCACGCACGAT
IFNa	CTGCTGGCTGTGAGGACATA	AGGAAGAGAGGGCTCTCCAG

Table 1 (continued).

Gene	Sequence (forward)	Sequence (reverse)
IFN β	CCCTATGGAGATGACGGAGA	ACCCAGTGCTGGAGAAATTG
IL-1 α	TCTATGATGCAAGCTATGGCTCA	CGGCTCTCCTTGAAGGTGA
IL6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TNF- α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
ICAM1	GGCATTGTTCTCTAATGTCTCCG	GCTCCAGGTATATCCGAGCTTC
VCAM	CCAAATCCACGCTTGTGTTGA	GGAATGAGTAGACCTCCACCT
TLR3	CTTGCGTTGCGAAGTGAAGAA	CCAATTGTCTGGAAACACCCC
RIG-I	ATTCAGGAAGAGCCAGAGTGTC	GTCTTCAATGATGTGCTGCAC
PKR	AAGCAGGAGGCAAGAAACG	TGACAATCCACCTTGTTTTCGT
MDA5	CGATCCGAATGATTGATGCA	AGTTGGTCATTGCAACTGCT
Flk1	GCAAAACACTCACCATTCCCA	GAGGTTTGAAATCGACCCTCG
Erg	ACCTCACCCCTCAGTCCAAA	TGGTCGGTCCCAGGATCTG
Fli1	ATGGACGGGACTATTAAGGAGG	GAAGCAGTCATATCTGCCTTGG
GATA2	CACCCCGCCGTATTGAATG	CCTGCGAGTCGAGATGGTTG
VE-cad	ATGAATCGCTGCCCCACTATG	CATTCCCTGTGTTAGCATCGAC
PECAM1	TGCACCCATCACTTACCACC	CTTCATCCACCGGGGCTATC
Oct-4	AGTTGGCGTGGAGACTTTGC	CAGGGCTTTCATGTCCTGG
Nanog	TTGCTTACAAGGGTCTGCTACT	ACTGGTAGAAGAATCAGGGTC
Sox-2	GACAGCTACGCGCACATGA	GGTGCATCGGTTGCATCTG
Klf4	GGCGAGTCTGACATGGCTG	GCTGGACGCAGTGCTTCTC
Rex-1	TGGAAGCGAGTTCCTTCTC	GCCGCCTGCAAGTAATGAG
GATA4	CCCTACCCAGCCTACATGG	ACATATCGAGATTGGGGTGTCT
GATA6	TTGCTCCGGTAACAGCAGTG	GTGGTCGCTTGTGTAGAAGGA
Brachyury	CAGCCCACCTACTGGCTCTA	GAGCCTCGAAAGAACTGAGC
Cdx2	CACCATCAGGAGGAAAAGTGA	CTGCGGTTCTGAAACCAAAT
FGF5	GCTGTGTCTCAGGGGATTGT	ACAGTCATCCGTAAATTTGGC
ISG15	AGGTCTTTCTGACGCAGACTG	GGGGCTTTAGGCCATACTCC
OAS1a	GCCTGATCCCAGAATCTATGC	GAGCAACTCTAGGGCGTACTG
IFNAR1	GACAACTACACCCTAAAGTGGAG	GCTCTGACACGAAACTGTGTTTT
IFNAR2	TGTCTGCGAGCCTAGAGACTA	AGCCGGGAATTTTCGTATTGTTAT
STAT1	GCTGCCTATGATGTCTCGTTT	TGCTTTTCCGTATGTTGTGCT
STAT2	CTGAAGGACGAACAGGATGTC	CAGGGTGGTTAATCGGCCAA
JAK1	ACGCTCCGAACCGAATCATC	GTGCCAGTTGGTAAAGTAGAACC
TYK2	TGCATCCACATCGCACACAA	CTCCTGGGGATTTCATGCCA
IRF9	TGTCTGGAAGACTCGCCTAC	GCAACATCCATACGACCTCTCT
SOCS1	CTGCGGCTTCTATTGGGGAC	AAAAGGCAGTCGAAGGTCTCG

Note. Sequences of all primers are listed from 5'-end to 3'-end.

IFN β and cytokine assays

The culture medium collected from treated cells was used to determine secreted IFN β and cytokines. IFN β was quantified with an ELISA kit that detects mouse IFN β (PBL Biomedical Laboratories) according to the manufacturer's instruction. IL6 and TNF α were analyzed with a Luminex cytokine assay kit (Millipore) and determined with a MAGPIX instrument. The data were analyzed according to the method described by Prabhakar et al. (Prabhakar, Eirikis, & Davis, 2002).

Cell proliferation, viability, and cell cycle analysis

Cell proliferation was determined by colony size and by cell number after toluidine blue (TB) staining as previously described (Guo, Chakraborty, Rajan, Wang, & Huang, 2010). The absorbance at 630 nm of TB stained cells was measured with a microtiter plate reader. The values, which correlate with the number of viable cells, were used as an indirect measurement of cell proliferation or viability. Cell cycle analysis by flow cytometry was performed after the cells were stained with 50 μ g/ml propidium iodide. The cell cycle profiles were generated with the CFlow software.

Protein analysis by flow cytometry

Cellular protein analysis by flow cytometry was performed according to our published method (Wang & Guo, 2012). Briefly, treated cells were incubated with the antibodies against the specific proteins to be analyzed. The cells were then incubated with secondary antibodies conjugated with FITC (fluorescein isothiocyanate) and examined by an Accuri C6 flow cytometer. The fluorescence intensity, which correlates with the

protein level, was determined with the CFlow software. In some experiments, cells were doubly stained with propidium iodide, so the expression level of the protein in different phases of the cell cycle could be simultaneously determined.

siRNA transfection

siRNAs targeting PKR and RIG-I or negative control siRNAs (Santa Cruz Biotechnology) were transfected to mESCs with a DharmaFECT reagent at a final concentration of 100 nM. The cells were then analyzed for siRNA knockdown efficiency or used for the experiments as specified.

Cell lysate preparation and Western blot analysis

Cells were directly lysed with a SDS sample buffer that contained 150 mM NaCl, 10 mM NaF, and 0.25 mM NaVO₄. Western blot analysis was carried out as previously described (Guo et al., 2010).

Immunostaining assay

Treated cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were washed with PBS containing 0.3% hydrogen peroxide to block endogenous peroxidase activity for 30 min. Cells were blocked with 2% normal goat serum with 0.4% Triton-X100 and incubated primary antibodies overnight at 4°. The cells were washed with PBS and then incubated with secondary antibodies conjugated with HRP (horseradish peroxidase) for 2 h at room temperature. Positive cells were detected using a TrueBlue detecting kit (KPL) under a phase contrast microscope.

Statistical analysis

Data were presented as the mean \pm SD derived either from three independent experiments or from a representative experiment performed in triplicates that were performed at least twice with similar results. Statistical analysis was performed using a 2-tailed and paired student's *t*-test. Differences are considered statistically significant when $P < 0.05$.

Results

mESCs are Susceptible to the Cytopathic Effects of LACV Infection

To investigate the responses of mESCs to viral infection, The author first infected cells with LACV (a negative sense ssRNA virus) that is known to cause lytic cell death of mammalian cells (Verbruggen et al., 2011). 10T1/2 cells, a mouse cell line with fibroblast properties (Haas & Tuan, 2000), were used as a positive control. Following the infection with LACV (MOI=1), cytopathic effects were observed at 48 h post infection in 10T1/2 cells. These effects included cell morphological changes such as rounding up and detachment from the culture dish. Similar effects were observed in mESCs at a longer incubation time (> 60 h) (Figure 5A, indicated by arrows). The viral infection of mESCs was confirmed by the expression of a LACV gene that encodes an M-segment protein (Gc protein) (Soldan, Hollidge, Wagner, Weber, & Gonzalez-Scarano, 2010). The infected cells were immunostained with monoclonal antibodies against the Gc protein (a gift from Dr. Samantha Soldan, University of Pennsylvania School of Medicine)

followed by flow cytometry analysis. As shown in Figure 5B, the expression of the Gc protein was detected at 30 h and was significantly increased at 40 h post infection.

The cytopathic effect of LACV was proportional to viral loading as indicated by the reduced number of viable cells at different MOIs (Figure 5C & D). LACV infection also caused cell cycle inhibition as indicated by an apparent reduction of cells in the G2 phase (Figure 5E, indicated by arrow) and a reduced expression level of cyclin E (Figure 5F). Therefore, the reduced cell number of mESCs by LACV infection could be due to the lytic cell death as well as by a general inhibition of cell proliferation associated with viral infection.

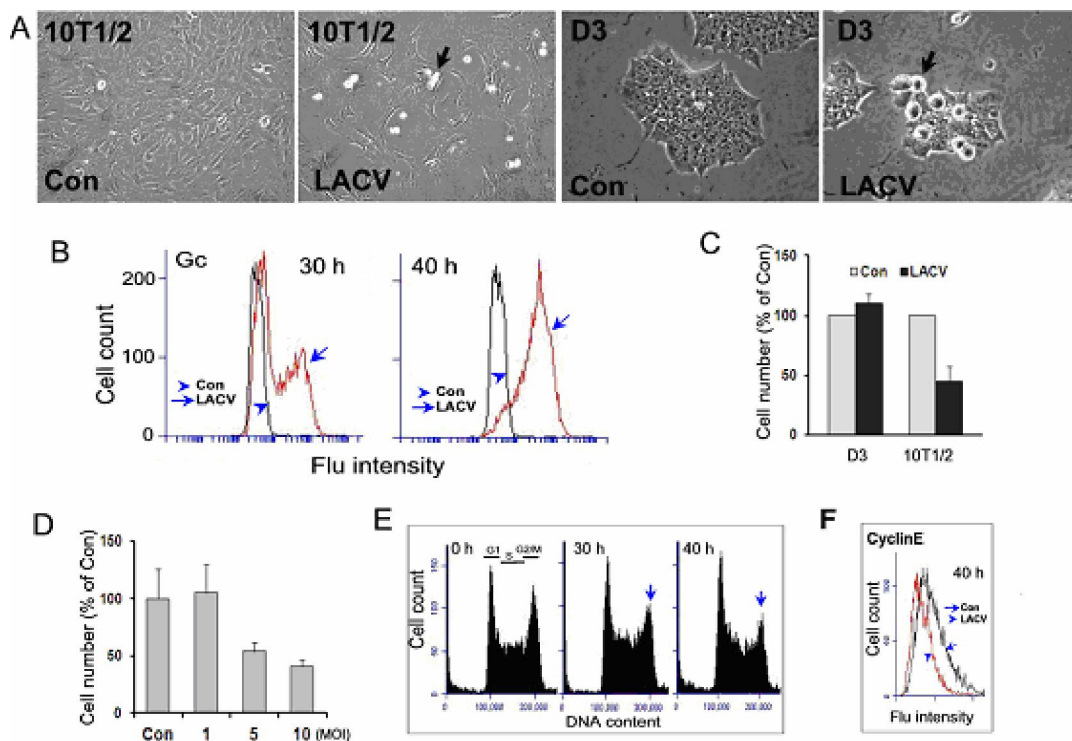


Figure 5. Infection of mESCs and 10T1/2 cells with LACV. **A**, mESCs (D3), and 10T1/2 cells were infected with LACV (MOI=1). The cells were examined under a phase contrast microscope and photographed (400 x) at 48 h for 10T1/2 cells and 60 h for D3 cells. Arrows denote detaching dead cells. **B**, Detection of LACV Gc protein in D3 cells infected with LACV (MOI=10) by flow cytometry. **C**, D3, and 10T1/2 cells were infected

with LACV at MOI of 1. Viable cells were determined at 48 h post infection by toluidine blue cell staining. The values are means \pm SD of a representative experiment performed in biological triplicates. D, D3 cells were infected with LACV at different MOI as indicated. Viable cells were determined at 48 h post infection as described in C. E, D3 cells were infected with LACV (MOI=10). The cell cycle profiles and cell count were determined with CFlow software. Reduction of the G2 phase cells was indicated by the arrow. F, Cyclin E expression level in LACV infected D3 cells described in E (40 h) was determined by flow cytometry. The control (Con) represents cells without viral infection. All experiments were performed at least twice with similar results.

mESCs are Deficient in Expressing Type I IFN in Response to Viral Infection

Under normal conditions, very low mRNA levels of IFN α and IFN β were detected in 10T1/2 cells and were nearly undetectable in mESCs. LACV infection induced about 1900-fold increase of IFN β in 10T1/2 at MOI of 1, but the induction in mESCs was negligible even at MOI of 10 (Figure 6A). In addition, LACV infection upregulated the expression of IFN β and the major viral RNA receptors, including RIG-I, PKR, MDA5, and TLR3, to different degrees in 10T1/2 cells, but such effects were not observed in mESCs (Figure 6A). These results indicated that mESCs are susceptible to LACV infection, but they are unable to effectively express IFN α and IFN β .

To determine if this observation is a general property of mESC or if it is an event specific to LACV infection, two additional viruses, WNV (a positive sense ssRNA virus) and SeV (a negative sense ssRNA virus), were tested. In both cases, viral infection induced a large increase of IFN β transcription in 10T1/2 cells, but the induction in mESCs was less than 5 fold (Figure 6B & C).

Viral infectivity in both mESCs and 10T1/2 cells was confirmed by the expression of viral proteins. The E glycoprotein encoded by WNV (Hanna et al., 2005) was detected with its monoclonal antibodies (4G2, ATCC) by immunostaining (Figure 6B, inset), while

the nucleocapsid protein (NP) encoded by SeV (Ye & Maniatis, 2011) was detected by RT-PCR analysis (Figure 6C, inset).

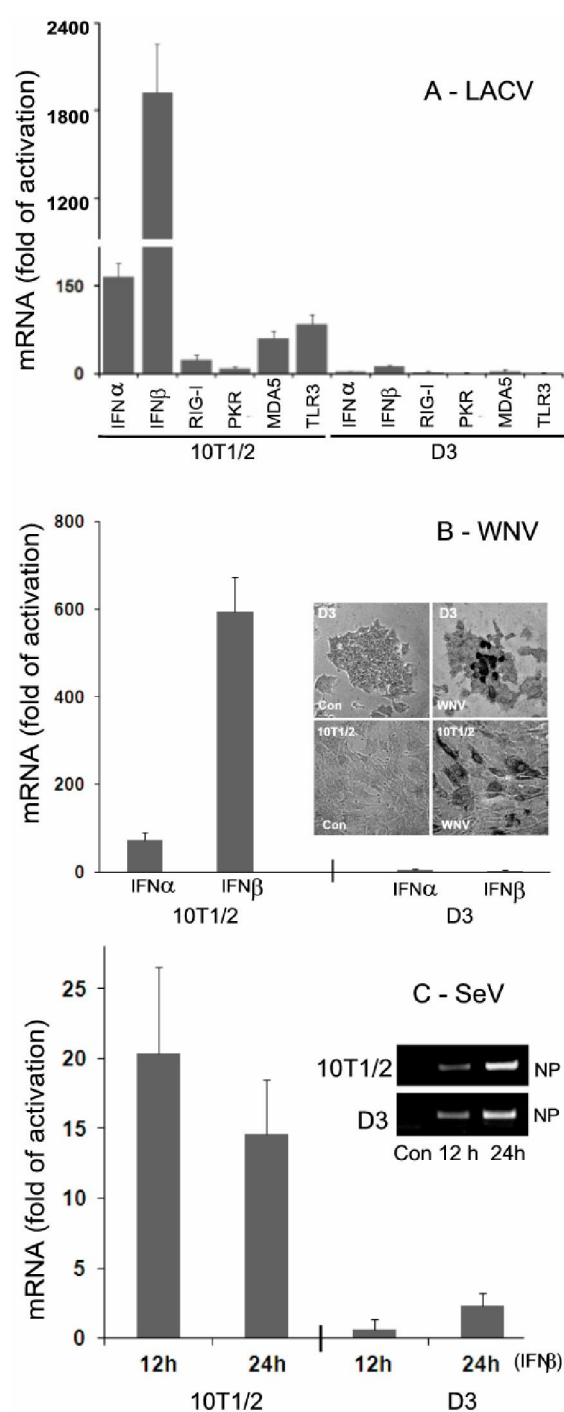


Figure 6. Viral infection induced expression of IFN α and IFN β in D3 and 10T1/2 cells. A, 10T1/2 and D3 cells were infected with LACV at MOI of 1 and 10, respectively. The mRNA levels of the tested genes were determined by RT-qPCR 24 h post infection. B,

10T1/2 and D3 cells were infected with WNV at MOI of 5 and 10, respectively. The mRNA levels of IFN α and IFN β were determined by RT-qPCR 24 h post infection (graph). The infected cells were immunostained with antibodies against a WNV E glycoprotein. The positive cells were detected as dark cells (inset). C, 10T1/2 and D3 cells were infected with SeV at 300 HAU/ml. The mRNA levels of IFN β were determined by RT-qPCR 12 h and 24 h post infection (graph). The transcription of SeV NP protein in infected cells was determined by RT-PCR and analyzed by agarose gel electrophoresis (inset). All results shown in the graphs are expressed as fold-activation where the mRNA level in control cells is designated as 1 (not shown). The values are means \pm SD of three independent experiments. The experiments shown in insets were performed twice with similar results.

Although a slight but consistent inhibition of cell proliferation was noted in the infected cells with WNV and SeV (data not shown), WNV and SeV did not cause apparent lytic cell death as LACV.

mESCs Can Sense and Respond to dsRNA via Cytosolic Receptors, but Fail to Produce Functional IFN β and IL6

The author further analyzed the responses of mESCs to polyIC, a synthetic analog of viral dsRNA that is widely used to mimic viral infection. When directly added to the cell culture, polyIC is expected to bind to TLR3 at the cell surface or to be internalized into the endosomes where it activates TLR3 (Matsumoto et al., 2003; Vercammen, Staal, & Beyaert, 2008). This phenomenon was demonstrated in Raw cells where polyIC induced the expression of IFN α and IFN β , but not in mESCs (data not shown), indicating that mESCs may not have functional TLR3 and/or a functional mechanism for polyIC internalization. However, when transfected into the cells, polyIC can induce several-fold increase of the transcription of three types of molecules typically seen in antiviral responses: type I IFN (IFN α/β), inflammatory cytokines (TNF α , IL1 α), and the cell adhesion molecule (VCAM1) in D3 mESCs (Figure 7A). Similar results were obtained in

another line of mESCs, DBA252 (Figure 7B). These results imply that mESCs can sense and respond to dsRNA via cytoplasmic receptors.

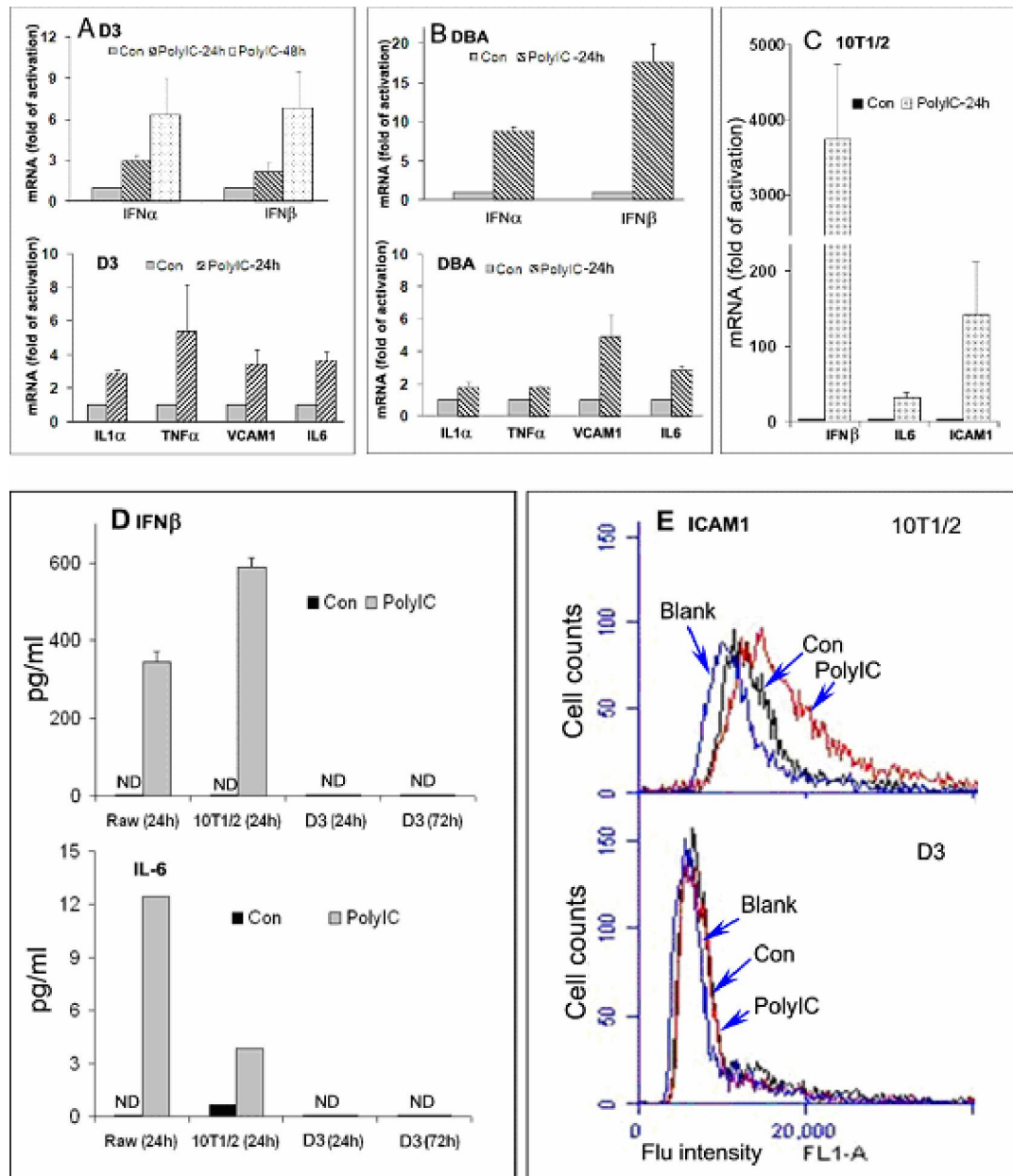


Figure 7. PolyIC transfected to mESCs can induce low level transcription of immune mediators but fail to produce detectable proteins. A, B, and C, D3, DBA252, and 10T1/2 were transfected with 300 ng/ml polyIC. mRNA was analyzed by RT-qPCR. The results are expressed as fold-activation where the mRNA level in control cells is designated as 1. The values in A and C are means \pm SD of three independent experiments. The values in B are means \pm SD of a representative experiment performed in biological triplicates. D, D3, and 10T1/2 were transfected with polyIC as described in A and C. For Raw cells, polyIC

(25 µg/ml) was directly added to the culture medium. The culture medium collected at different time points was analyzed for IFN β by ELISA (detectable range 15.6-1000 pg/ml) and IL6 by Luminex assay (detectable range 0.64-10000 pg/ml). ND, not detected. The values for IFN β are means \pm SD of a representative experiment performed in triplicate. The values for IL6 are average of a representative experiment performed in duplicate. E, Expression profiles of ICAM1 in 10T1/2 and in D3 cells determined by flow cytometry. The representative experiments in all panels were repeated at least twice with similar results.

However, the induction of IFN β in mESCs was negligible when compared with 10T1/2 cells, whereby polyIC-induced nearly a 4000-fold increase of IFN β mRNA. PolyIC-induced transcription of IL6 and ICAM1 was also substantially higher in 10T1/2 cells than in mESCs (Figure 7C). When examined at the protein level by ELISA, neither IFN β nor IL6 was detected in the media collected from polyIC-transfected mESCs, whereas they were readily detected in the samples from Raw cell and 10T1/2 cell cultures (Figure 7D).

Similar results were obtained for TNF α (data not shown). Similarly, ICAM1 protein was induced by polyIC transfection in 10T1/2 cells, but not in mESCs, as analyzed by flow cytometry of the cells immunostained with anti-ICAM1 antibodies (Santa Cruz) (Figure 7E). Therefore, transfected polyIC could elicit a limited increase of transcripts of the above mentioned immune mediators in mESCs, but their corresponding proteins were not detected.

PolyIC-transfected to mESCs Inhibits Cell Proliferation

The most notable effect of transfected polyIC on mESCs at the cellular level was the inhibition of cell proliferation. As shown in Figure 8, the colonies of polyIC-transfected cells were much smaller than the control colonies (Figure 8A),

correlating with markedly reduced cell numbers (Figure 8B). In control cells, about 60% of cells were in the S phase, but polyIC caused an apparent reduction of cells in the S and G2/M phases (Figure 8C, indicated by arrow and arrowhead, respectively). A time course analysis revealed that the effects of polyIC can be detected as early as 6 h (Figure 8D). It is noted that the polyIC transfection also caused a small number of apoptotic cells (floating cells, data not shown), which may also contribute to the reduced cell number and colony size.

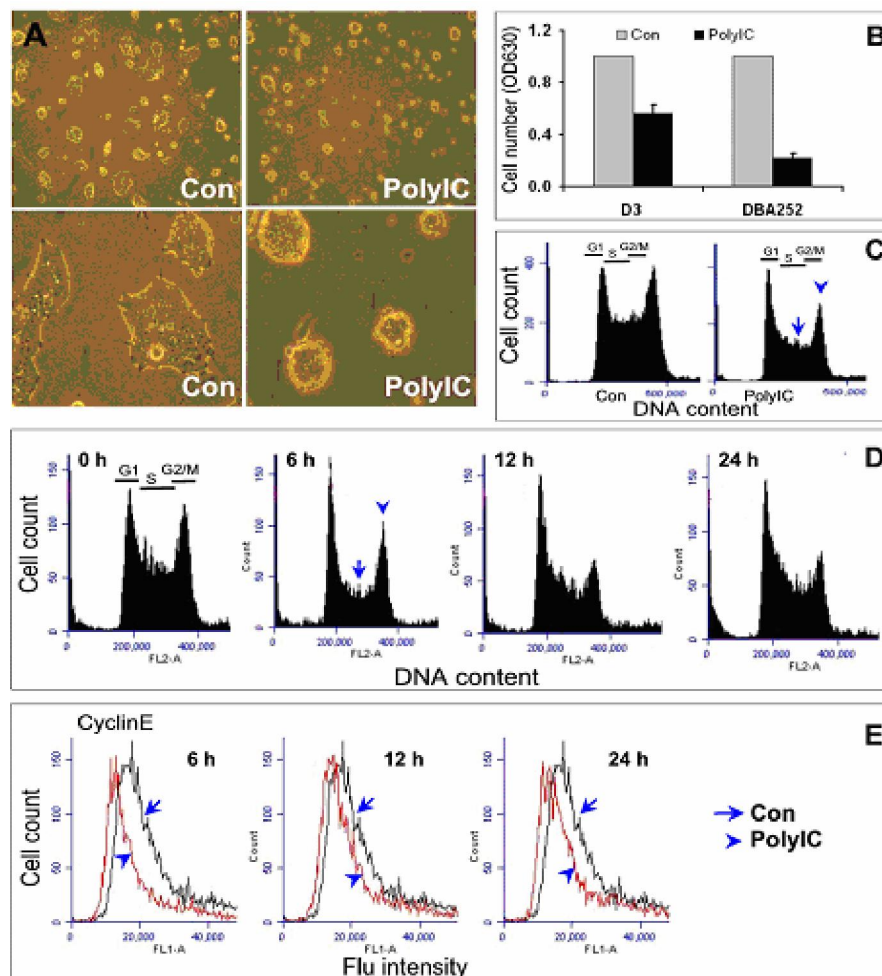


Figure 8. Effects of polyIC on mESC colony formation and cell proliferation. A-C, D3 cells were transfected with 300 ng/ml polyIC. Control (Con) represents cells transfected with DharmaFECT without polyIC. A, After incubation for 40 h, the colonies were

examined under a phase contrast microscope and photographed with a digital camera (100 x, upper panels; 400 x, lower panels). B, Cell proliferation was measured by cell number indirectly determined from toluidine blue staining (absorbance at 630 nm). The values are means \pm SD of an experiment performed in biological triplicate. C, Cell cycle was analyzed by flow cytometry. The cell cycle profiles and cell count were determined with a computer software. Reduction of the S and G2/M phase cells were indicated by the arrow and arrowhead, respectively. D, D3 cells were transfected with polyIC and incubated for the times as indicated. The cell cycle progression was analyzed by flow cytometry as described in C. E, The effect of polyIC on cyclin E expression was determined by flow cytometry after immunostaining of the cells with antibodies against cyclin E. All representative experiments were performed at least twice with similar results.

Since the constitutive activation of Cdk2 by cyclin A and E is the key factor that drives the rapid cell proliferation in mESCs (Stead et al., 2002), the effects of polyIC on the expression of these cell cycle regulators were examined.

The reduced expression of cyclin E was apparent at 6 h after treatment, and this trend lasted for 24 h, as determined with its antibodies (Santa Cruz) by flow cytometry (Figure 8E). Although cyclin A was not affected at 6 h and 12 h, it was reduced at 24 h. A similar pattern was seen for Cdk1 and Cdk2. The CdkIs, p16, p19, and p21, were expressed at very low levels in control cells, and polyIC did not affect their expression (data not shown). These results suggest that down-regulation of cyclin E, and to a lesser extent, cyclin A, Cdk1, and Cdk2, contributed to polyIC-inhibited cell proliferation.

The Effect of polyIC on the Stem Cell State

To test if the stem cell state of mESCs was affected by polyIC, I examined the mRNA levels of pluripotency markers in polyIC-transfected cells. They were transiently down-regulated at 6 h and 12 h, but recovered nearly to the level of the controls by 24 h (Figure 9A).

In agreement with mRNA change, the protein level of Oct4, a key gene in the pluripotency control network (Niwa, 2007), displayed a similar pattern with an initial slight decrease, but returned to the normal levels at 24 h (Figure 9B).

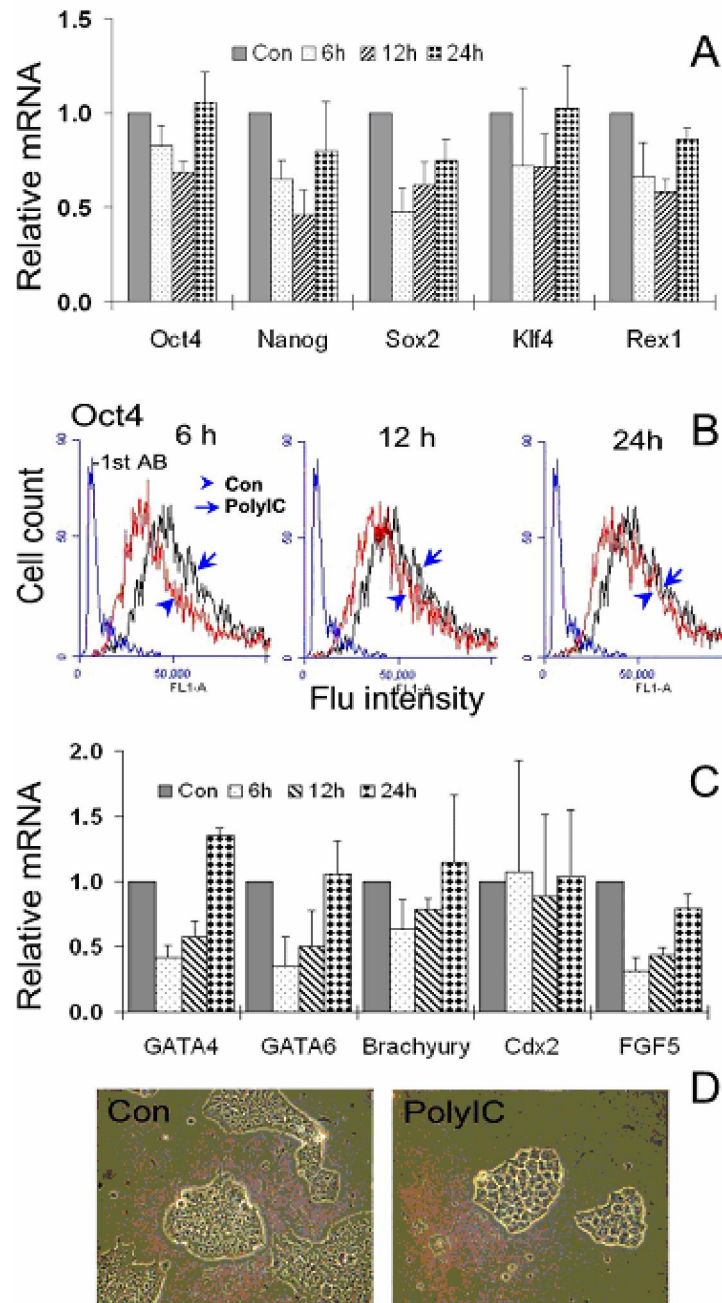


Figure 9. Effects of polyIC on the expression of pluripotency/differentiation markers and cell/colony morphology of mESCs. D3 cells were transfected with 300 ng/ml polyIC and incubated for a time course of 24 h. A and C, The mRNA levels of pluripotency markers

and early differentiation markers were determined by RT-qPCR. The results are means \pm SD of three independent experiments. B, The effect of polyIC on the protein level of Oct 4 was determined by flow cytometry. -1st AB represents the experiments where the cells were not incubated with anti-Oct4 antibodies (1st antibodies) as the control. D. The effect of polyIC on cell/colony morphology was examined under a phase contrast microscope and photographed with a digital camera at 72 h after treatment (400x). The images are representative experiments performed at least three times.

Interestingly, the transcription of several early differentiation markers showed a similar dynamic pattern (Figure 9C) to the pluripotency markers (Figure 9A).

Due to polyIC-induced cell cycle inhibition, the colonies formed from polyIC-transfected cells were smaller than those formed from control cells, but their overall morphology (round with densely packed undifferentiated cells) was not significantly changed, as examined at 40 h (as shown in Figure 9A) or at 72 h post transfection (Figure 9D).

The Relative Expression Levels of dsRNA Receptors in mESCs and 10T1/2 cells

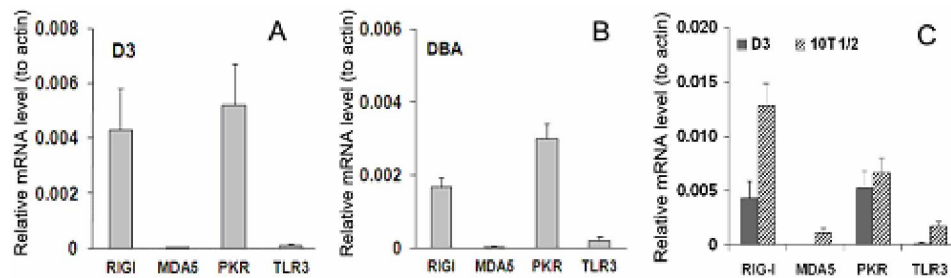


Figure 10. Relative mRNA levels of dsRNA receptors in mESCs and 10T1/2 cells. The mRNA levels of dsRNA receptors in D3 (A), DBA252 (B), and 10T1/2 (C) were determined by RT-qPCR. Their relative abundance in each cell was determined by comparing with the mRNA level of β -actin (designated as 1). For a direct comparison, the relative mRNA level of each molecule in D3 cells and 10T1/2 cells was presented in panel C. The results are means \pm SD of three independent experiments in all panels.

To identify the receptor(s) that mediate the effects of transfected polyIC, the relative expression levels of the four dsRNA receptors was analyzed. The mRNA level of each molecule was determined by RT-qPCR and compared with that of β -actin. In D3

cells, TLR3 is expressed at a very low level, and MDA5 is barely detected, while RIG-I and PKR are expressed at higher levels (Figure 10A). A similar pattern was seen in DBA252 cells (Figure 10B). When compared with 10T1/2 cells, the mRNA of TLR3 and MDA5 in D3 cells are negligible, while RIG-I is about one third of that in 10T1/2 cells. However, PKR is expressed at similar levels in the two types of cells (Figure 10C).

PKR Plays a Key Role in Mediating the Effects of polyIC in mESCs

The higher basal expression levels of PKR and RIG-I in mESCs make them the likely candidates that mediate the effects of polyIC. The author first investigated PKR by its ability to phosphorylate eukaryotic initiation factor 2 α (eIF2 α). It is known that phosphorylation of eIF2 α by PKR attenuates its function in translation, thereby resulting in protein synthesis inhibition (Garcia, Meurs, & Esteban, 2007). PolyIC indeed stimulated eIF2 α phosphorylation in mESCs as determined by flow cytometry and confirmed by Western blot analysis using antibodies that specifically recognize phosphorylated eIF2 α (p-eIF2 α Cell Signaling) whereas, the expression of PKR did not change significantly. p-eIF2 α was detected as early as 30 min after polyIC transfection (Figure 11A, boxed areas), which preceded cell cycle inhibition. Pretreatment of the cells with a PKR inhibitor C16 (Sigma) blocked polyIC-induced eIF2 α phosphorylation, indicating that PKR is the responsible kinase. These results were confirmed with siRNA targeting PKR, which attenuated eIF2 α phosphorylation while the negative control siRNA had no effect (Figure 11B, upper panels, boxed areas). Importantly, silencing PKR reversed polyIC-induced reduction of S and G2/M phase cells (Figure 11B, middle

panels) as well as reduced colony size (Figure 11B, lower panels). On the other hand, knocking down RIG-I did not show such effects. These data demonstrate that PKR, not RIG-I, plays a key role in mediating polyIC-induced cell cycle inhibition.

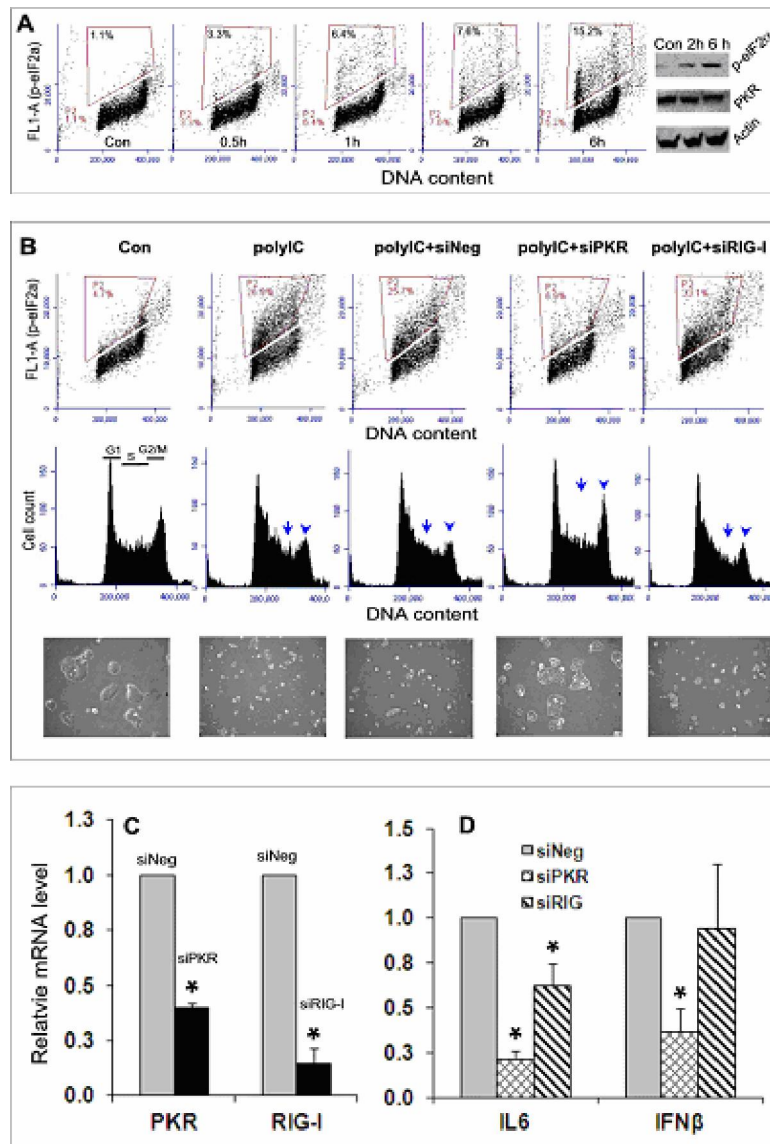


Figure 11. PKR plays key roles in mediating the effects of polyIC. **A**, PolyIC-induced phosphorylation of eIF2 α . D3 cells were transfected with 300 ng/ml polyIC and incubated for times indicated. The cells with phosphorylated eIF2 α (p-eIF2 α) were quantified by flow cytometry (boxed areas. White slash lines were used to help identify the bottom sides of the boxes). The blot insets illustrate the levels of p-eIF2 α and PKR determined by Western blot. Actin was used as a loading control. **B**, The effects of PKR and RIG-I silencing on polyIC-induced cellular events. D3 cells were transfected with

siRNA against PKR (siPKR), RIG-I (siRIG-I), or negative control siRNA (siNeg) for 24 h. The cells were split into new dishes. After 24 h, the cells were transfected with polyIC for 24 h and analyzed for p-eIF2 α by flow cytometry (upper panels), cell cycle by flow cytometry (middle panels, the changes in S and G2/M phase cells were indicated by the arrow and arrowhead, respectively), and cell proliferation/colony formation by microscopy (lower panels). C and D, Relative contributions of PKR and RIG-I to polyIC-induced mRNA of IL6 and IFN β . D3 cells were transfected with siPKR, siRIG-I, or siNeg followed by polyIC treatment as described in B. The mRNA levels of PKR and RIG-I (C) or IL6 and IFN β (D) were determined by RT-qPCR and compared with the mRNA in the cells that were transfected with siNeg (defined as 1) in each group. The results are means \pm SD of three independent experiments. The difference is considered to be statistically significant when $p < 0.05$ (*). All representative experiments in A and B were performed at least twice with similar results.

The effects of silencing PKR and RIG-I on polyIC-induced transcription of IFN β and IL6 were then analyzed. siRNA targeting PKR and RIG-I specifically knocked down their targets as expected (Figure 11C). Silencing PKR significantly reduced mRNA levels of both IL6 and IFN β while silencing RIG-I slightly attenuated transcription of IL6, but not that of IFN β (Figure 11D).

PKR is Activated by LACV, but not by WNV or SeV Infection in mESCs

Whether or not PKR was activated in viral infected cells was analyzed. As shown in Figure 12A, a time course study of LACV-infected cells indicated PKR was activated at 11 h post infection and lasted up to 30 h as indicated by eIF2 α phosphorylation, while the protein level of PKR was not altered as determined by Western blot with PKR antibodies (Santa Cruz).

Knocking down PKR expression did not have an apparent effect on LACV-induced cell death (Figure 12B). Similar results were obtained in the experiments with C16 PKR inhibitor (data not shown). PKR activation by LACV was further confirmed by flow cytometry with antibodies against p-eIF2 α ; however, p-eIF2 α was not

detected in the cells infected with WNV or SeV in parallel experiments (Figure 12C).

These results suggested that PKR activation is virus-dependent in mESCs.

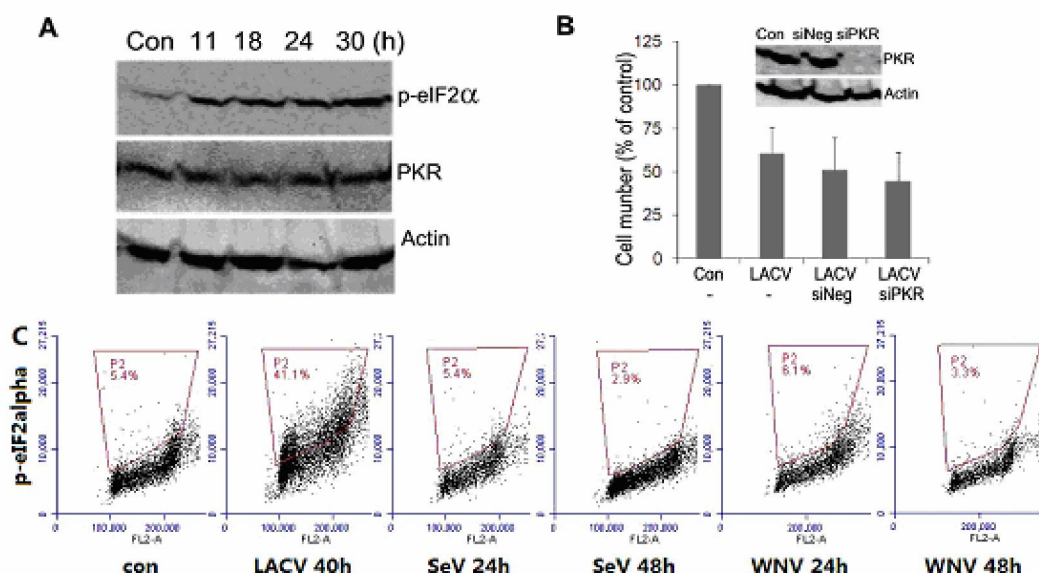


Figure 12. Effects of viral infections on PKR activation. **A**, Activation of PKR by LACV infection in mESCs. D3 cells were infected with LACV (MOI=10) for the different time periods as indicated. Activation of PKR was analyzed by Western blot with antibodies against p-eIF2 α . Actin was used as a control for protein loading. **B**, Silencing PKR did not have an apparent effect on LACV-induced cell death. D3 cells were transfected with siRNA against PKR (siPKR) or control siRNA (siNeg) as described in Figure 7C. The efficiency and specificity of PKR knockdown were analyzed by Western blot (blot inset) after 36 h transfection. Con represents the cells without transfection. The cells, either transfected with siRNA (siNeg or siPKR) or without (Con), were infected with LACV at MOI of 10. The cell viability was determined at 48 h post infection as described in Figure 1. **C**, WNV and SeV infection did not activate PKR in mESCs. D3 cells were infected with WNV (MOI=10) or SeV (300 HAU/ml) for the specified times. The cells with p-eIF2 α were quantified by flow cytometry (boxed areas. White slash lines were used to help identify the bottom sides of the boxes). Cells infected with LACV (MOI=10) were used as a positive control for comparison. The values shown in the graph (B) are means \pm SD of three independent experiments. The results in all other panels are representatives of experiments performed at least twice with similar results.

Discussion

In the first project, the responses of mESC to three types of live viruses and two synthetic viral dsRNA analogs were investigated. While all of these stimuli induced a

robust IFN α/β expression in 10T1/2 cells, they only induced very limited or no detectable transcription of IFN α/β . These results suggest that mESCs are deficient in type I IFN expression, a central component of the antiviral mechanisms in most types of somatic cells.

It is known that many viruses have developed certain mechanisms to avoid the host antiviral responses (Haller, Kochs, & Weber, 2006). Repressing IFN α/β induction in host cells is one of such mechanisms that have been reported for LACV and SeV (Haller et al., 2006; Ye & Maniatis, 2011). However, the capacity of these viruses to repress IFN α/β induction seems to depend on the types of host cells, as demonstrated in 10T1/2 cells in which LACV can induce strong transcription of IFN α/β as well as the key components involved in IFN α/β expression, such as RIG-I, MDA5 and TLR3. The induction of IFN β by WNV and SeV in 10T1/2 cells was also significant. Therefore, the failure to express IFN α/β in mESCs is not virus-specific or due to the viral anti-IFN induction mechanism. This conclusion is in line with the observations recently reported by Wash et al. (Wash et al., 2012) that herpes simplex virus type I (HSV-1, a DNA virus) and influenza virus exert (a negative sense ssRNA virus) cytopathic effects without evoking type I IFN induction. Together with results from the experiments with polyIC and 3p-ssRNA (see CHAPTER IV), which mimic viral RNA in inducing a robust IFN α/β expression in 10T1/2 cells but not in mESCs, it can be concluded that deficiency in type I IFN expression is an intrinsic property of mESCs.

This study provided the molecular basis that explains the defective IFN α/β expression in mESCs. In some cell types, polyIC can induce IFN α/β via activation of TLR3 at the cell surface or in the endosomes (Meylan & Tschopp, 2006; Nasirudeen et al., 2011; Stetson & Medzhitov, 2006). When transfected into the cells, polyIC can induce robust IFN α/β expression and other responses that are similar to those evoked by viral infection via cytoplasmic dsRNA receptors (Fortier et al., 2004; Matsumoto & Seya, 2008). mESCs were unresponsive to polyIC that was directly added to the medium, likely due to the very low level expression of TLR3. However, polyIC transfected into the mESCs showed a profound inhibitory effect on proliferation, a known effect of polyIC on many types of differentiated cells, indicating that the cytoplasmic receptors for polyIC are active in mESCs.

One of the distinctive features of mESCs is their rapid cell proliferation that is driven by constitutively active Cdk2/cyclin A/E complexes (Burdon et al., 1999; Singh & Dalton, 2009). It is conceivable that actively dividing mESCs are more sensitive to polyIC-inhibited translation since cell proliferation critically depends on *de novo* protein synthesis. PolyIC inhibited cell proliferation was expected to significantly affect the stem cell state. However, the morphology of the individual cells and colonies appeared to be maintained at the undifferentiated state despite their transient decrease in transcription of pluripotency markers. Since polyIC caused similar changes to the transcription of differentiation markers, the effect is not gene specific, but rather a general transcription disturbance similar to that caused by viral infection (Garcia et al., 2007). It is speculated

that the non-specific transcription interference might help mESCs maintain their stem cell state by preventing the expression of genes that promote differentiation, at least for a short period of time under our experimental conditions. PolyIC-induced cell cycle inhibition is transient since polyIC-treated cells can resume a normal rate of proliferation in the subculture when they were passed to new culture dishes. Thus, the transient change of pluripotency and differentiation markers is not surprising since the experiments were performed in the presence of leukemia inhibitory factor (LIF), which maintains the pluripotency and prevents differentiation of mESCs. This observation is in line with the author's recent study demonstrating that the transient inhibition of cell proliferation does not compromise the stem cell state of mESCs (Wang & Guo, 2012).

It is generally believed that MDA5 and RIG-I play primary roles in mediating viral RNA induced IFN α/β expression in the cytoplasm (Nasirudeen et al., 2011; Stetson & Medzhitov, 2006) while PKR also contributes to and modulates this process (Garcia et al., 2007; Samuel, 2001). Since MDA5 is expressed at negligible level in mESCs, it is conceivable that PKR and/or RIG-I may mediate the effects of transfected polyIC. However, the RIG-I signaling pathway seems to be inactive since silencing RIG-I did not affect the effects of polyIC and that 3p-ssRNA (5'-triphosphate single-stranded RNA), the best studied ligands of RIG-I (Pichlmair et al., 2006; Yoneyama & Fujita, 2007), failed to induce IFN β in mESCs (see Chapter IV). On the other hand, substantial evidence indicates that PKR plays a key role in mediating the effects of polyIC, as demonstrated by the phosphorylation of eIF2 α and the results observed in the PKR silencing experiments.

It is known that IFN-induced activation of ribonuclease L (RNase L) can hydrolyze both cellular and viral RNA (Samuel, 2001). Although polyIC could lead to RNase L activation in some cells, this does not seem to be the case in mESCs since polyIC did not cause rRNA degradation (data not shown), which is a commonly used indication of RNase L activation (Castelli et al., 1997). The result further supports the conclusion that polyIC could not induce a sufficient level of IFN α/β in mESCs for RNase L activation.

LACV infection also activated PKR in mESCs, similar to the finding in murine embryonic fibroblasts (MEFs) (Streitenfeld et al., 2003). However, silencing or inhibition of PKR did not significantly affect LACV-induced cell death of mESCs, which is in line with the observation that PKR activation has little impact on viral replication as demonstrated in PKR^{-/-}MEFs (Streitenfeld et al., 2003). PKR activation has been implicated in apoptosis, but its roles are stimulus- and virus-dependent (Zhang & Samuel, 2007). The effects of PKR activation by LACV on mESCs could be more complex than polyIC due to the fact that many other cellular and viral factors are involved during infection. Unlike LACV infection, PKR activation was not detected in the mESCs infected with WNV or SeV. Failure to activate PKR by these viruses has been seen in other cells. As recently reported in rodent cells, WNV infection does not induce PKR activation, possibly through a mechanism that sequesters viral dsRNA from host PKR (Elbahesh, Scherbik, & Brinton, 2011). In SeV, it is proposed that PKR activation is repressed by the C protein encoded by the virus (Takeuchi, Komatsu, Kitagawa, Sada, & Gotoh, 2008). Whether or not they utilize these mechanisms in mESCs remains to be investigated.

It is interesting to note that hESCs do not express IFN α/β even at the mRNA level when transfected with polyIC (Chen et al., 2010; Foldes et al., 2010). mESCs can express limited transcripts of IFN α/β , IL6, and ICAM1, but nonetheless could not produce detectable levels of their respective proteins. Thus, the transcription of these immune mediators in mESCs may have limited function, if any, as an antiviral mechanism. In speculating the physiological significance of dsRNA induced cellular events in ESCs, it is worthy of mentioning that in differentiated cells, dsRNA can be formed under some circumstances, but they are retained in the nucleus for editing and rarely exported to the cytoplasm (Zhang & Carmichael, 2001). However, the nuclear retention mechanism in ESCs is less effective. As a result, dsRNAs may be exported to the cytoplasm in ESCs where they activate dsRNA receptors (Chen & Carmichael, 2009). In addition, other types of abnormal RNAs, such as those derived from cell death or from RNA processing, could activate PKR (Amarante & Watanabe, 2010). In particular, PKR is considered to be a “sentinel kinase for stress” for its involvement in stress response in addition to antiviral response (Williams, 1999, p. 6112). Therefore, it appears that dsRNA-induced inhibition of translation and cell proliferation in mESCs could be an important mechanism that helps cells deal with immunogenic stresses.

In summary, the data demonstrate that the expression of type I IFN as a crucial part of antiviral responses is underdeveloped in mESCs. This study may open up an important area in ESC research for understanding the development of antiviral mechanisms during embryogenesis and how the stress from immunogenic signals affects ESC physiology.

CHAPTER IV

MOUSE EMBRYONIC STEM CELLS HAVE UNDERDEVELOPED ANTIVIRAL MECHANISMS THAT CAN BE EXPLOITED FOR THE DEVELOPMENT OF mRNA-MEDIATED GENE EXPRESSION STRATEGY

Introduction

The data from the previous chapter demonstrated that mESCs do not express type I IFNs in response to viral infections and double-stranded RNA, but they are susceptible to LACV-induced lytic cell death and polyIC-induced cytotoxicity (a synthetic analog of viral dsRNA) (Wang et al., 2013). In this chapter, the author have further investigated the responses of mESCs to synthetic single-stranded RNA (ssRNA) and protein-encoding mRNA, which mimic viral RNA in inducing antiviral responses.

The landmark achievement in generating induced pluripotent stem cells (iPSCs) has led to the new concept of cell reprogramming (Takahashi & Yamanaka, 2006), but the fact that viral vectors are commonly used for effective expression of reprogramming factors prevents the therapeutic use of the reprogrammed cells (Buganim, Faddah, & Jaenisch, 2013; Muller, Daley, & Williams, 2009). Extensive effort to avoid this problem has led to the development of several alternatives, among which mRNA-mediated gene expression has shown great promise due to its non-integrating nature (Daubman, 2011). This method directly introduces synthetic mRNA into the host cell for the expression of reprogramming factors, thus eliminating the need for viral or DNA vectors. The successful generation of RiPSCs (RNA-induced iPSCs) from fibroblasts (Angel & Yanik,

2010; Plews et al., 2010; Warren et al., 2010; Yakubov, Rechavi, Rozenblatt, & Givol, 2010) has led to the belief that this strategy is the beginning of the new era of cell reprogramming (Daubman, 2011). This strategy could in principle be expanded to reprogram any type of cell as long as the genes that control the cell fate are identified. A major biological challenge, however, is that synthetic mRNA is detected as a viral RNA analog by host cells and induces strong antiviral responses resulting in interferon induction, protein synthesis inhibition, and reduced viability of host cells (Hornung et al., 2006; Pichlmair et al., 2006). Synthetic mRNA must therefore be modified via a complex process to minimize their effects in eliciting antiviral responses (known as immunogenicity) (Warren et al., 2010). The lack of antiviral responses in mESCs prompted the author to investigate the feasibility of developing an mRNA-based gene expression strategy, with the expectation that mESCs would allow effective translation of synthetic mRNA without suffering the adverse effects associated with antiviral responses encountered in differentiated somatic cells.

ESCs have attracted enormous attention in recent years for their differentiation potential as a cell source for regenerative medicine (Wobus & Boheler, 2005). Methods for the differentiation of ESCs to specific cell lineages have developed, most of which primarily depend on the spontaneous differentiation potential of ESCs and the influence of certain growth factors and/or cytokines. For example, VEGF and bFGF are commonly used to stimulate endothelial cell differentiation (Levenberg, Golub, Amit, Itskovitz-Eldor, & Langer, 2002; McCloskey, Stice, & Nerem, 2006; Yamashita et al.,

2000). However, the efficiency of these methods is usually so low that isolating a pure cell population is difficult. The lack of effective differentiation methods to obtain specific types of cells in sufficient quality and quantity is a major challenge that limits clinical applications of ESC-derived cells. Cell lineage specification is mainly driven by the activation of cell-specific transcription factors during early embryogenesis. It is conceivable that the inefficient differentiation of existing methods is, at least in part, due to insufficient transcription activation during *in vitro* differentiation. A recent study reported that hESCs can be effectively differentiated into endothelial cells by viral vector-mediated expression of transcription factors that control vascular differentiation (Ginsberg et al., 2012), demonstrating that intervention at the transcription level can provide a much stronger driving force for ESC differentiation into a specific cell fate.

In this study, mESCs were shown to be able to tolerate repeated transfection and effectively express synthetic mRNA with expected biological functions without the need for any chemical modifications of synthetic mRNA. Therefore, by exploiting the underdeveloped antiviral mechanisms in mESCs, the author demonstrated the feasibility that mRNA-mediated gene expression could be developed into novel strategies that manipulate gene expression in ESCs.

Methods

Cell culture and treatment

ESCs (D3 cell line, ATCC) were cultured in standard mESC medium that contains leukemia inhibitory factor (LIF) as previously described (Wang et al., 2013).

Epithelial cells (SKOV3 cells, a human ovarian carcinoma cell line) and mouse fibroblasts (10T1/2 cell line, derived from mouse embryonic tissues that can differentiate into several cell lineages) were cultured in RPMI and DMEM medium, respectively, which contain 10% fetal calf serum and 100 units/ml penicillin and 100µg/ml streptomycin. All cells were maintained at 37 °C in a humidified incubator with 5% CO₂ (Darland & D'Amore, 2001; Haas & Tuan, 2000).

Cells were plated at 40-50% confluence and were usually cultured for 24 h before the experiments. Synthetic dsRNA (polyIC [polyinosinic-polycytidylic acid], Sigma), ssRNA, and mRNA in different formats were transfected into the cells with DharmaFECT reagent (Thermo Scientific) as previously described (Wang et al., 2013). The control cells were transfected with DharmaFECT reagent only. The cells were collected and used for various analyses after incubation for the times indicated in the individual experiments.

In vitro synthesis and purification of short single-strand RNA (3p-ssRNA) and synthetic mRNA

For preparation of short random sequenced 3p-ssRNA and HO-ssRNA, the dsDNA templates for *in vitro* transcription were prepared by the primer extension of the DNA oligo containing the T7 φ2.5 promoter sequence (5'-CGTAATACGACTTCACTATTAG) with a DNA template (5'-N_nCTAATAGTGAGTCGTATTACG, N = random nucleotide sequence, A, G, C, T, n=18, 38, 58, or 78) using Taq DNA polymerase (Promega). The resulting dsDNA was

ethanol-precipitated and used as templates for *in vitro* transcription with the Ampliscribe T7-Flash Transcription Kit (Epicentre) to produce 5'-3p ssRNA (5'-pppAGN_n, n=18, 38, 58, or 78, the resulting RNA are termed 3p-20nt, 3p-40nt, 3p-60nt, and 3p-80nt) following the manufacturer's protocol. The RNA transcripts were ethanol-precipitated and purified by 8% denaturing polyacrylamide gel electrophoresis (PAGE). HO-ssRNA was prepared by removing the 5' triphosphate of above 3p-ssRNA with calf intestinal alkaline phosphatase (Takara) followed by PAGE purification. The resulting RNA are named as HO-20nt, HO-40nt, HO-60nt, and HO-80nt.

For preparation of protein-encoding mRNA, the DNA template for enhanced green fluorescence protein (EGFP) mRNA was generated by PCR from a pEGFP-N1 plasmid (BD Biosciences) using two primers containing sequences of 5'-CGTAATACGACTCACTATTAGGAAGCTTCGAATTCTGCAGTCG and 5'-TTTACGCCTTGAGATACATTGATG. The DNA template for mouse *Etv2* was amplified by PCR from cDNA library prepared from a mouse lung endothelial cells using primers with the sequences of 5'-CCGTAATACGACTCACTATTAGGAACCGTCAGAACAAGCATCC and 5'-TTTTGTTTTTGTGTTTTTGTGTTTTATTGGCC. PCR was performed with Q5 High-Fidelity DNA polymerase (New England Biolabs). The resulting dsDNA templates contain the T7 ϕ 2.5 promoter for *in vitro* transcription, the 5'-UTR region with the Kozak sequence, and the open reading frame of EGFP or *Etv2*. In addition, EGFP has a 3'-UTR region.

Using the above EGFP DNA as the template, 3p-EGFP-mRNA (long 3p-ssRNA) was prepared by *in vitro* transcription under the same condition as the short ssRNA preparation described previously and was purified by an M100 Microcon membrane filter. To prepare functional EGFP and Etv2 mRNA, *in vitro* transcription from their DNA templates was carried out in the presence of the cap analog m⁷GpppA (chemically synthesized in our lab, unpublished) to generate 5'-capped EGFP and Etv2 mRNA transcripts. The purified RNA transcripts were polyadenylated by *E. coli* Poly(A) polymerase (New England Biolabs), resulting in functional mRNA, m⁷GpppA-EGFP-polyA, and m⁷GpppA-Etv2-polyA.

Microscopic analysis and quantitative measurement of GFP expression

The expression of GFP in live cells was visualized using a LSM confocal fluorescence microscope. To quantitatively measure the GFP expression level, the cells were grown in a black-walled, clear-bottom 96-Well cell culture plate (Costar) and analyzed with a SpectraMax® M5 Scanning Multi-Detection Microplate Reader (Molecular Devices) according to the manufacture's instruction.

Reverse-transcription Real-time quantitative polymerase chain reaction (RT-qPCR), cell proliferation, viability, and cell cycle analysis, protein analysis by flow cytometry, and Western blot, and statistical analysis were described in the Method part of Chapter III.

Results

Characterization of Short Synthetic ssRNA-induced Antiviral Responses in Epithelial Cells

Synthetic short ssRNA and long protein-encoding mRNA prepared by *in vitro* transcription have been used as viral RNA mimics to study antiviral responses. The length and the 5'-triphosphate (3p) group of RNA are considered to be the most important factors in eliciting antiviral responses while the sequence seems to be less critical (Gondai, Yamaguchi, Miyano-Kurosaki, Habu, & Takaku, 2008; Schlee et al., 2009a; Wang et al., 2010). A panel of short 3p-ssRNA of 20, 40, 60, and 80 nucleotides (nt) with a random sequence of A, U, C, and G were synthesized, each of which was detected as a single band with expected size by PAGE analysis (Figure 13A, gel inset).

The potency of these short ssRNA in eliciting antiviral responses was first determined in a human ovarian epithelial carcinoma cell line (SKOV3 cells), which have been previously used as a model to study the immunogenicity of different types of RNA (Kubler et al., 2010; Kubler et al., 2011; Van et al., 2012). Using IFN β expression as a benchmark for antiviral responses, it showed that only 3p-ssRNA with longer than 60 nt induced IFN β , while the removal of the 3p group completely abolished this effect, as demonstrated in the case of 3p-80nt-ssRNA (Figure 13A, 3p-80nt vs HO-80nt). 3p-60nt-ssRNA and 3p-80nt-ssRNA caused cell death (Figure 1B & C), in parallel with their potency of IFN β induction. The cytotoxicity was not seen when the cells were treated with HO-80nt-ssRNA, correlating with its inability to induce IFN β (Figure 13A).

Similar results were observed with a ssRNA of 145 nt that encodes a segment of luciferase (data not shown). These results are in line with the characteristics of synthetic ssRNA previously described by other investigators (Hornung et al., 2006; Kim et al., 2004; Pichlmair et al., 2006).

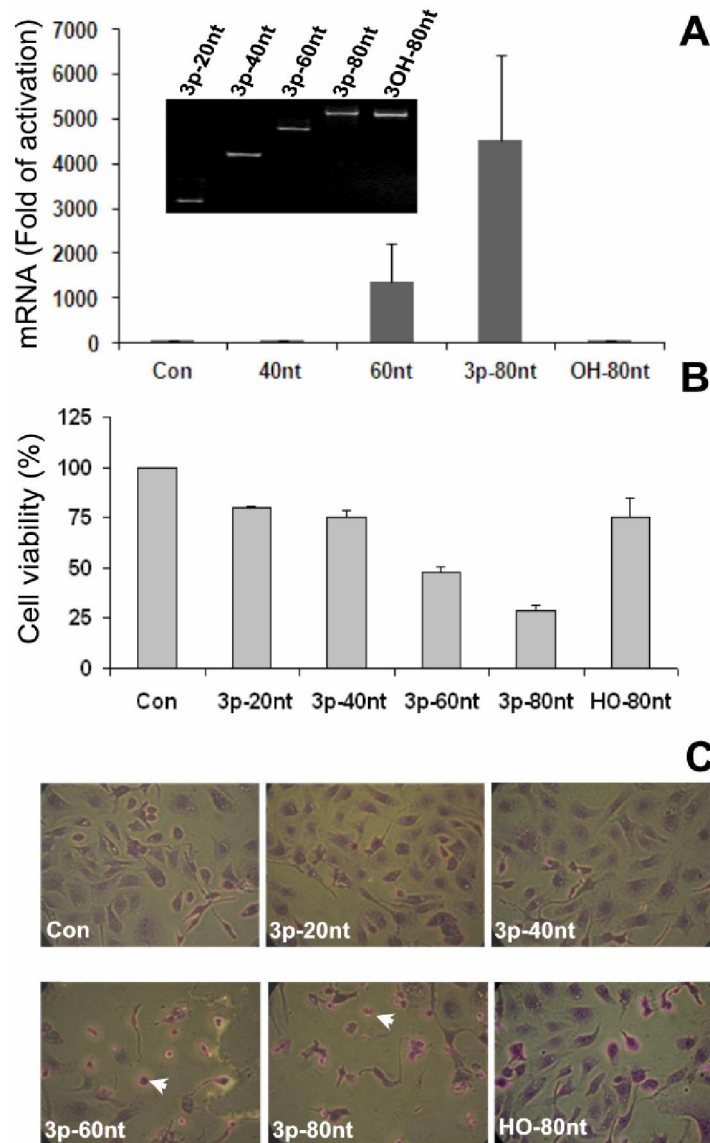


Figure 13. ssRNA induced antiviral responses in SKOV3 cells. The cells were transfected with a different format of ssRNA (40nM) as indicated. A, The induction of IFN β mRNA were determined by RT-qPCR at 12 h post transfection. Control (Con) represents cells transfected with DharmaFECT without ssRNA. The results are means \pm SD of three independent experiments. The gel inset shows the PAGE analysis of purified

ssRNA. B, Cell viability was determined by toluidine blue cell staining of viable cells at 24 h post transfection. The values are means \pm SD of a representative experiment performed in triplicates. C, Transfected cells were examined under a phase contrast microscope (200 x) at 48 h after toluidine blue cell staining. The images were photographed from a representative experiment. Arrows denote apoptotic cells. All representative experiments were performed at least twice with similar results.

Short 3p-ssRNA Induced Strong Antiviral Responses in Fibroblasts but not in mESCs

Using 3p-80nt-ssRNA characterized in SKOV3 cells, its effects on 10T1/2 cells were investigated, which are fibroblasts derived from early mouse embryonic tissues with mesenchymal stem cell properties that can differentiate into several cell lineages (Darland & D'Amore, 2001; Haas & Tuan, 2000). Similar to SKOV3 cells, 3p-80nt-ssRNA induced strong IFN β expression in these cells, as well as the expression of several other genes commonly upregulated during antiviral responses, including IL-6 (an inflammatory cytokine), ICAM-1 (a cell adhesion molecule), and RIG-I and MDA5 (cytosolic RNA receptors) (Figure 14A, a). 3p-80nt-ssRNA also reduced cell viability in a dose-dependent manner (Figure 14A, b). The cytotoxicity caused by 3p-80nt-ssRNA was apparent at 12 h after transfection and significantly increased at 24 h as indicated by cell death and detachment (Figure 14A, c).

A time course analysis indicated that IFN β induction by 3p-80nt-ssRNA was detectable at 7 h and dramatically increased at 14 h and 20 h post transfection (Figure 14A, d), coinciding with the onset of cytotoxicity and increased cell death. However, when mESCs (D3 cells) were treated with 3p-80nt-ssRNA under the same conditions, the induction of IFN β was negligible at 12 h treatment (Figure 14B, a) in comparison with 10T1/2 cells and was not detected at 6 h or 24 h time points (data not shown). The

upregulation of IL-6 (Figure 14B, a) ICAM-1, MDA5, and RIG-I was not observed in D3 cells (data not shown). Undifferentiated ESCs are characterized by their rapid cell proliferation with about 60% of cells in S phase and their growth in compact colonies. Transfection with 3p-80nt-ssRNA did not have detectable effects on cell viability, colony morphology, or cell cycle profile (Figure 14B, b, c, or d, respectively). Similar results were obtained when the cells were treated with 3p-80nt-ssRNA up to 100 μ M (data not shown). The different responses in 10T1/2 cells and D3 cells were not due to the different transfection efficiency since both cell types effectively expressed transfected GFP mRNA as will be discussed later.

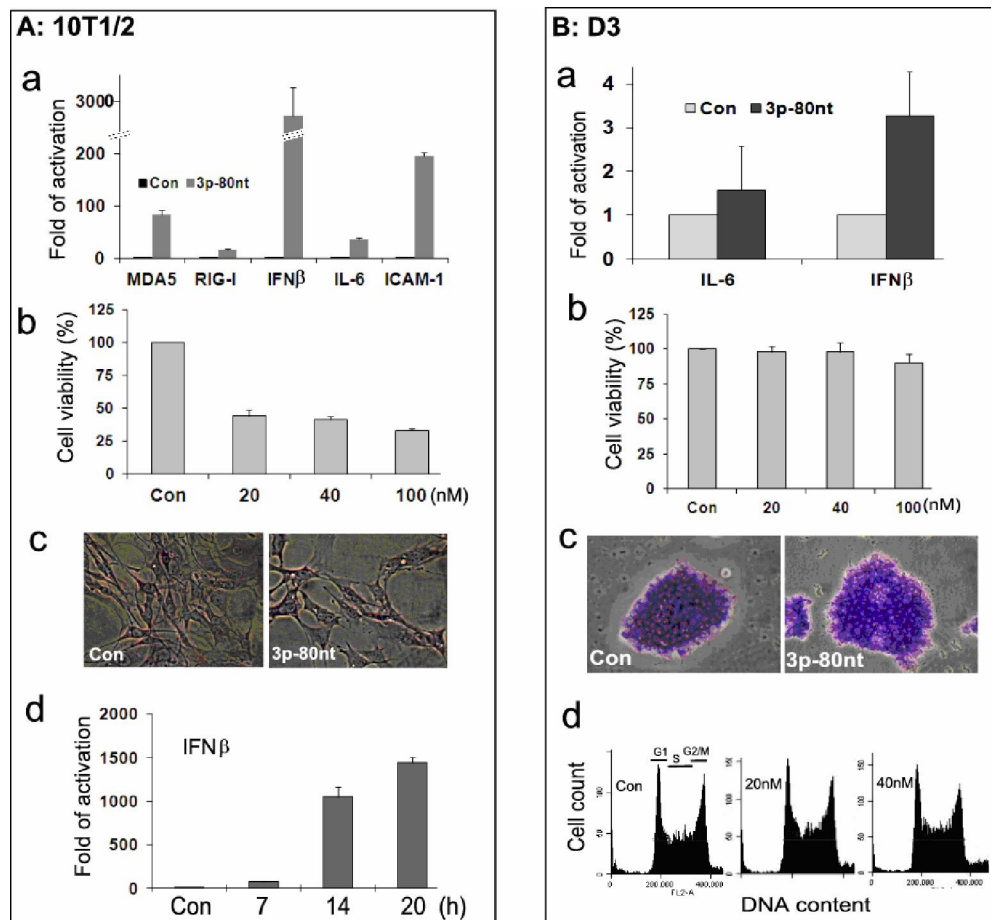


Figure 14. Comparative analysis of 3p-ssRNA induced antiviral responses in 10T1/2 cells and D3 cells. The cells were transfected with 3p-80nt-ssRNA (40 nM). The mRNA levels of IFN β and other gene as indicated were determined by RT-qPCR in cells transfected for 12 h (a). The results are means \pm SD of three independent experiments. The cell viability was determined by toluidine blue cell staining of viable cells at 24 h post transfection of (b). The values are means \pm SD of a representative experiment performed in triplicates. A time course of IFN β mRNA induction in 10T1/2 cells by 3p-80nt-ssRNA (40 nM) was determined by RT-qPCR. The values are means \pm SD of a representative experiment performed in triplicates (A, d). The cell cycle profiles and cell count of D3 cells were determined with CFlow software at 24 h post transfection (Bc). The morphology of transfected 10T1/2 cells (Ac) and D3 cells (Bd) were examined under a phase contrast microscope (400 x) after TB cell staining. All representative experiments were repeated at least twice with similar results.

Protein-encoding mRNA (long ssRNA) Induced Antiviral Responses in Fibroblasts and mESCs

To test the effects of long ssRNA, we used the coding sequence of EGFP mRNA with a 3p group (3p-EGFP, 1033 nt, without cap and polyA tail). It not only strongly induced the expression of IFN α and IFN β in 10T1/2 cells (~150 fold and ~5500 fold, respectively), but also upregulated RNA receptors (RIG-I, TLR3 and PKR) that play key roles in antiviral responses (Figure 15A), and caused cell death (data not shown, similar to the effects of 3p-80nt-ssRNA illustrated in Figure 14A, b and c). On the other hand, 3p-EGFP mRNA only induced about 5 and 10 fold increase of IFN α and IFN β respectively in D3 cells (Figure 15B), which is negligible in comparison with its effects on 10T1/2 cells. The 3p-EGFP mRNA did not have significant impact on cell viability or the morphology of D3 cells (data not shown, as illustrated in Figure 14B, b and d).

It is noted that unlike 3p-80nt-ssRNA where the removal of the 3p group completely abolished its immunogenicity as shown in Figure 13, the removal of the 3p group from EGFP mRNA significantly reduced, but did not completely abolish IFN β

induction since HO-EGFP mRNA can still induce ~40 fold increase of IFN β in 10T1/2 cells. These observations were in line with the notion that the 3p group plays a critical role in type I IFN induction, but the length also contributes the immunogenicity of ssRNA (Hornung et al., 2006; Pichlmair et al., 2006; Schlee et al., 2009b).

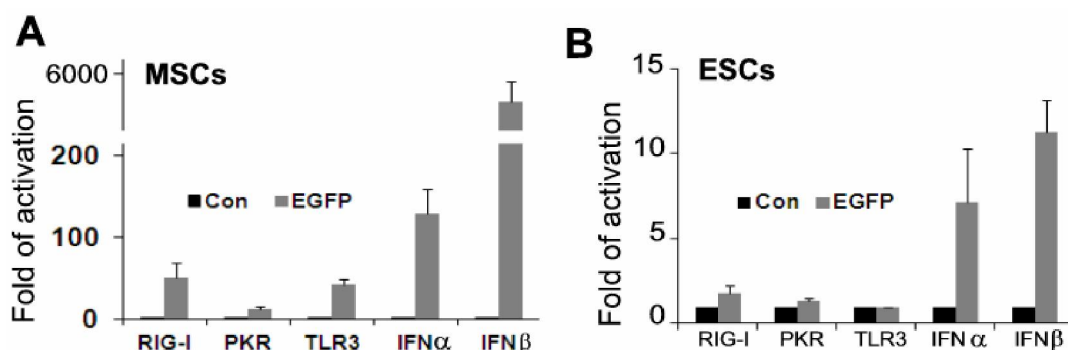


Figure 15. Comparative analysis of EGFP-mRNA-induced antiviral responses in 10T1/2 cells and D3 cells. The cells were transfected with 3p-EGFP-mRNA (EGFP, 300 ng/ml). The mRNA levels of genes involved in antiviral response were determined by RT-qPCR. The results are means \pm SD of three independent experiments.

dsRNA, but not ssRNA, Activates PKR in mESCs

dsRNA can directly activate PKR (dsRNA-activated protein kinase), which in turn phosphorylates eukaryotic initiation factor 2 α (eIF2 α) and results in a general inhibition of translation and cell proliferation, thereby limiting viral replication as a part of antiviral mechanisms (Garcia et al., 2007; Samuel, 2001). It is noted that polyIC fails to induce type I IFN in D3 cells, but it can activate PKR and induce a strong inhibition of cell proliferation (Wang et al., 2013). Since it has been reported that PKR can also be activated by ssRNA that have certain structural features (Nallagatla, Toroney, & Bevilacqua, 2011), the author analyzed the effect of 3p-EGFP mRNA on PKR activation in mESCs. As a positive control experiment, the transfection of D3 cells with polyIC significantly increased the level of phosphorylated eIF2 α , indicating the activation of

PKR (Figure 16A, polyIC); however, transfecting the cells with 3p-EGFP mRNA did not show any effect as compared with the control experiment (Figure 16A, EGFP mRNA).

It has been previously demonstrated that PKR activation by polyIC significantly inhibited the synthesis of cyclins, thereby inhibiting D3 cell proliferation (Wang et al., 2013). While this effect of polyIC-induced PKR activation is reflected by the reduced cell number in cell proliferation analysis (Figure 16B) and by the decreased cell population at G2M phase in cell cycle analysis (Figure 16C), the effects of 3p-EGFP mRNA on these cellular processes are rather limited, if there is any.

mESCs can Effectively Express Synthetic mRNA with Expected Biological Functions

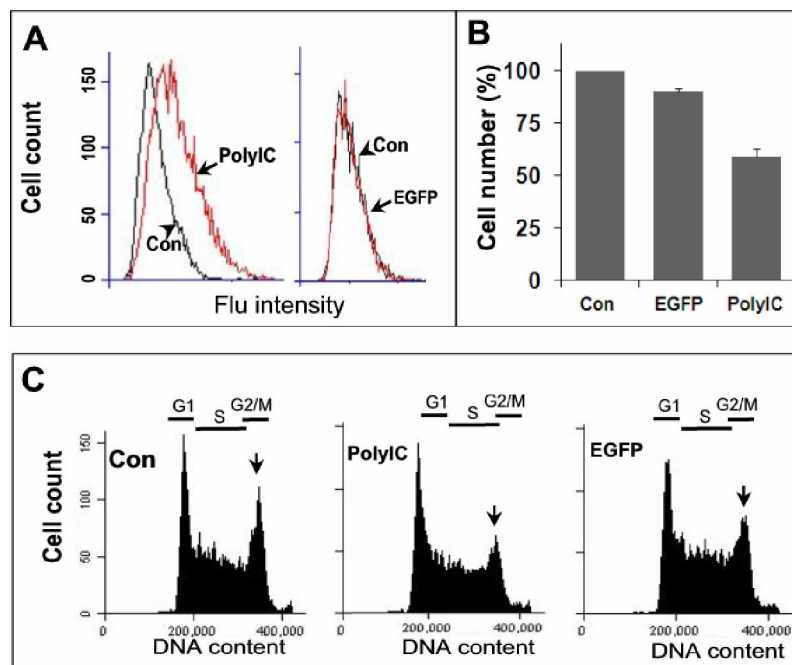


Figure 16. Effects of polyIC and 3p-EGFP-mRNA on PKR activation and cell proliferation. D2 cells were transfected with 300 ng/ml 3p-EGFP-mRNA (EGFP mRNA, coding sequence) or polyIC. A, The activation of PKR was determined by the increase of phosphorylated eIF2 α (indicated by fluorescence intensity) with flow cytometry after immunostaining of the cells with antibodies against phosphorylated eIF2 α . Cell proliferation was determined by TB staining. The values are means \pm SD of a representative experiment performed in triplicate. C, Cell cycle progression was analyzed

by flow cytometry. Reduction of G2/M phase cells was indicated by the arrows. All experiments were repeated at least twice with similar results.

To determine if synthetic mRNA can be translated in the cells, a functional EGFP was produced by adding a cap and a polyA tail to EGFP-mRNA (m^7GpppA -EGFP-polyA).

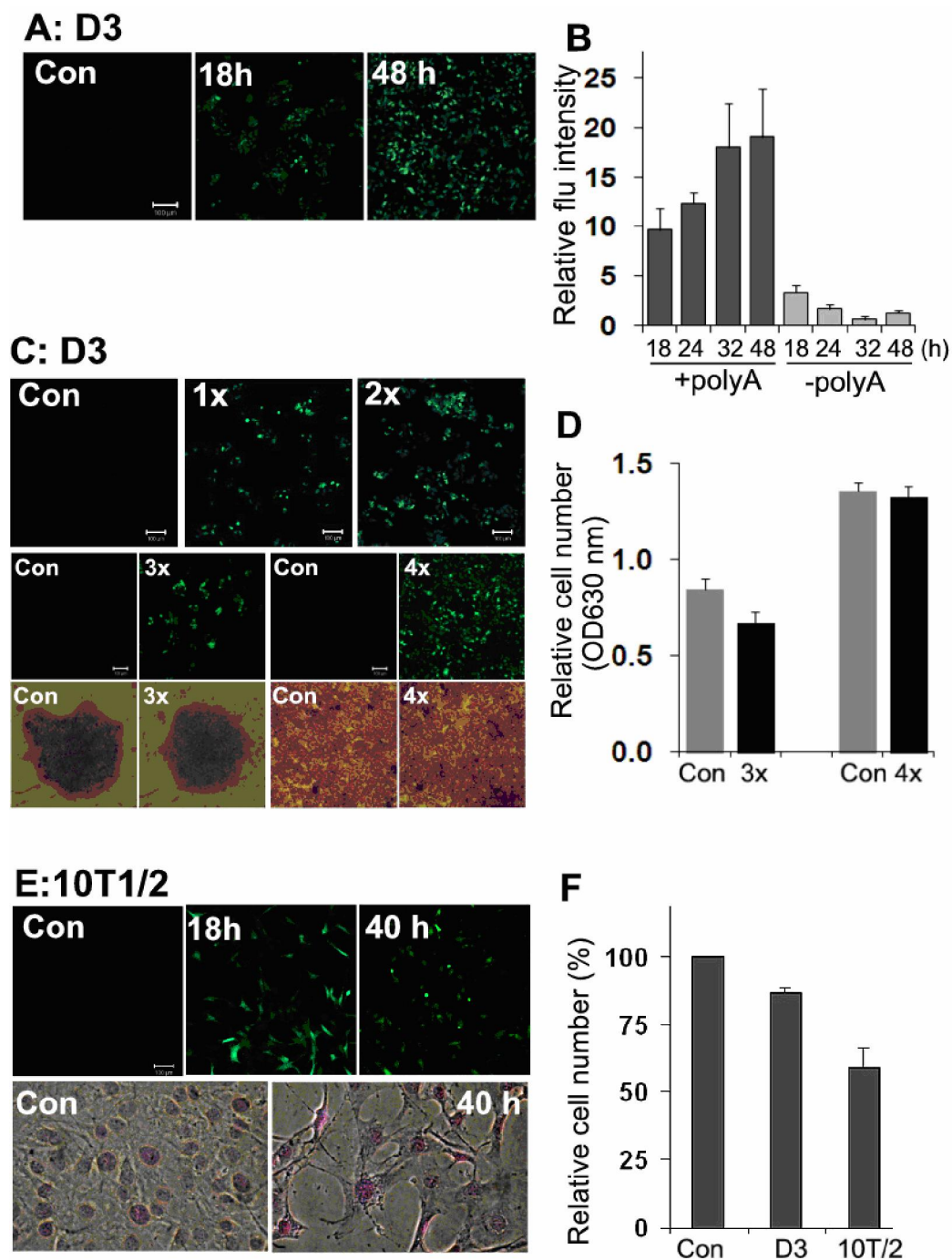


Figure 17. Expression of EGFP in 10T1/2 cells and D3 cells from synthetic mRNA. The cells were transfected with 300 ng/ml of m⁷GpppA-EGFP-polyA (+polyA). Control (Con) cells were transfected with DharmaFECT without mRNA. A, The expression of EGFP in D3 cells was examined with a fluorescence microscope at 18 h and 48 h after one time transfection. B, The expression of EGFP in D3 cells from m⁷GpppA-EGFP-polyA (+polyA) or m⁷GpppA-EGFP (-polyA) was quantified with a fluorescence plate reader within a time course from 18-48 h. C, D3 cells were transfected with m⁷GpppA-EGFP-polyA once or twice (1x & 2x, respectively). The expression of EGFP was examined with a fluorescence microscope at 24 h of each transfection. The cells after second transfection were split into new culture dishes, where they received third (3x) and fourth transfection (4x) following the same protocol used for the first two rounds of transfections. The cells were examined for EGFP expression with a fluorescence microscope (middle panels) and for cell morphology with a phase contrast microscope (bottom panels). D, The number of viable cells (determined by OD630 nm) was determined by TB staining at 24 h after third (3x) and fourth (4x) transfection. E, 10T1/2 cells were transfected m⁷GpppA-EGFP-polyA under the same conditions as described for D3 cells and examined for EGFP expression and for cell morphology. F, The relative number of viable cells was determined by TB staining at the end of the experiment (40 h for 10T1/2 cells and 48 h for D3 cells for comparison). The values are means \pm SD of a representative experiment performed in triplicates. All experiments were repeated at least twice with similar results.

After transfection, EGFP expression was detected in D3 cells as early as 12 h, and the fluorescence intensity progressively increased in a time course from 18 h to 48 h as determined by fluorescence microscopy and by spectrometric analysis (Figure 17A& B), whereas very limited EGFP expression was detected with an EGFP mRNA without a poly A tail (m⁷GpppA-EGFP) (Figure 17B, -polyA). In a separate experiment, D3 cells grown at low density were transfected with m⁷GpppA-EGFP-polyA, and were then transfected a second time after 24 h. The expression of EGFP was analyzed under a fluorescence microscope at 24 h after each transfection. EGFP was effectively expressed after each transfection with a significant increase of EGFP fluorescence intensity after the second transfection (Figure 17C, 1x vs 2x). After second transfection, the cells quickly reached confluence. Therefore, they were split into new culture dishes where they received third

and fourth transfection. Similar to the first two rounds of transfection, EGFP was effectively expressed under both low (Figure 17C, 3x) and high density (Figure 17C, 4x) growth conditions. The transfection of ESCs grown in low density slightly decreased the cell number, but such effect was not obvious in cells at a high density (Figure 17D, 3x and 4x, respectively). These results demonstrated that ESCs can tolerate repeated mRNA transfection and effectively translate the synthetic mRNA without losing cell viability.

When 10T1/2 cells were transfected with m⁷GpppA-EGFP-polyA, EGFP was detected at 18 h, similar to its expression in D3 cells. However, the cells began to lose viability around 30 h after transfection, concurrent with a decrease in fluorescence signal. EGFP expression was significantly diminished with only about 60% of cells remaining viable as compared with the control cells at 40 h (Figure 17E, F). Therefore, even after being capped and polyadenylated, the functionalized mRNA can still cause a strong toxicity in 10T1/2 cells, although the synthetic mRNA can be translated at an early stage of transfection before the cells mount strong antiviral responses.

To further determine the functionality and compatibility of synthetic mRNA with mESCs, D3 cells were transfected with the Etv2 mRNA (m⁷GpppA-Etv2-polyA), a member of the ETS transcription factor family that directly transactivates several genes required for vascular development (De Val & Black, 2009; Ginsberg et al., 2012). As shown in Figure 18, several genes essential for endothelial cell specification (known target genes of Etv2) were upregulated to different degrees in D3 cells transfected with Etv2 mRNA (Figure 18A), demonstrating the expected transcription activity of Etv2.

However, the three genes most upregulated in D3 cells, Flk1, Fli1, and VE-cadherin (VE-Cad), were not significantly affected in 10T1/2 cells (Figure 18B). Similar to EGFP mRNA, Etv2 mRNA transfection did not affect the viability of D3 cells, but caused significant cytotoxicity in 10T1/2 cells (Figure 18C & D).

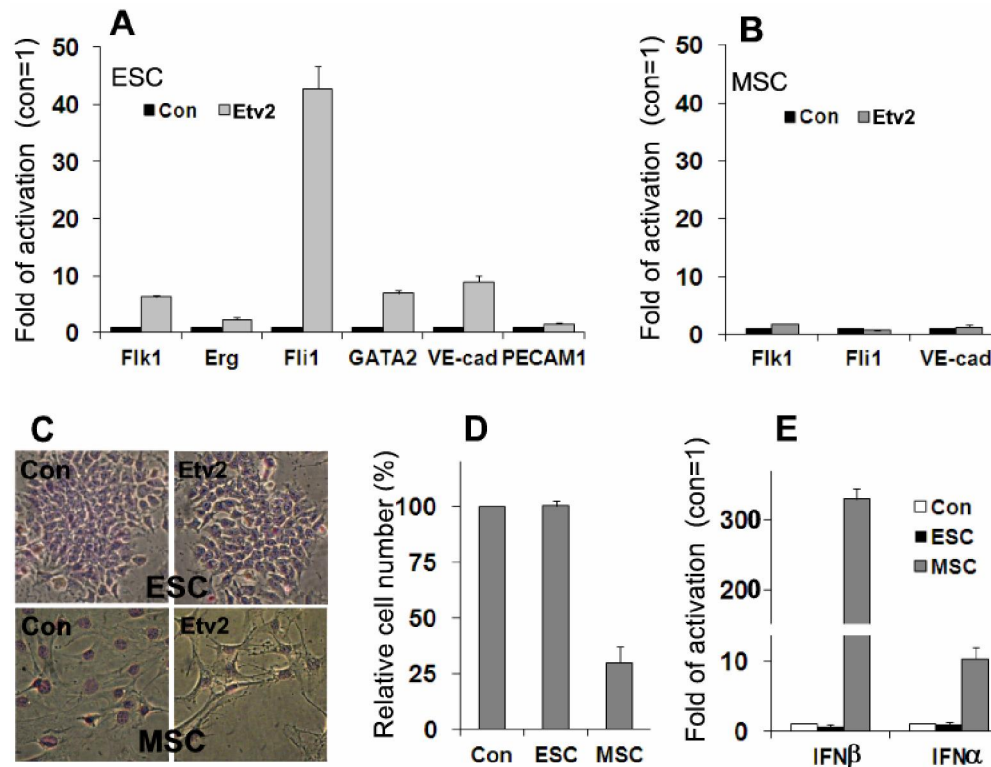


Figure 18. Expression of Etv2 from its synthetic mRNA in 10T1/2 cells and D3 cells. The cells were transfected with m⁷GpppA-Etv2-polyA (300 ng/ml) for 48 h. A, B, The effects on the expression of Etv2 regulated genes were determined by RT-qPCR analysis. The results are means \pm SD of three independent experiments. C, The morphology of transfected cells were examined under a phase contrast microscope (400x) after TB cell staining. D, The relative number of viable cells was determined by TB staining at the end of the experiment. The values are means \pm SD of a representative experiment performed in triplicates that was repeated at least twice with similar results.

These results correlated with the strong induction of IFN α/β by Etv2 mRNA in 10T1/2 cells, but not in D3 cells (Figure 18E). Therefore, the failure of Flk1, Fli1, and VE-cadherin expression in 10T1/2 cells can be logically explained by the fact the antiviral responses, especially PKR activation and cytotoxicity, are known factors to

strongly inhibit protein synthesis in the host cells (Garcia et al., 2007; Samuel, 2001). It is conceivable that the antiviral responses induced by Etv2 mRNA in 10T1/2 cells will inhibit its own translation, resulting in no transcription activation required for the target genes (Flk1, Fli1 and VE-cadherin).

Chemical Modifications Reduce, but not Eliminate the Immunogenicity of Synthetic mRNA

The 3p group is an intrinsic part of RNA synthesized by *in vitro* transcription (Huang, He, Zhang, & Guo, 2008). When the mRNA is capped and polyadenylated, the functionalized synthetic mRNA still can induce strong antiviral responses as shown in 10T1/2 cells, and other investigators have reported in other types of fibroblasts (Angel & Yanik, 2010). Base modifications of the mRNA during *in vitro* transcription have been used to reduce the immunogenicity. However, cell reprogramming requires repeated transfection. Therefore, the modified mRNA must be used in combination with either siRNA knocking down the RNA receptors in the host cells or inhibitors of IFN signaling pathway (Warren et al., 2010). To test how mESCs respond to mRNA modifications, a fully functional GFP mRNA with base modifications (purchased from Stemgent) was used. It also contains a nuclear localization signal sequence that directs the translated GFP to the nucleus (designated as nGFP-mRNA). The nGFP mRNA was effectively translated in both 10T1/2 cells and D3 cells and detected in the nucleus as expected after transfection for 24 h (Figure 19A, 1x). The cells did not display detectable cytotoxicity within 48 h of transfection. However, subsequent transfection for the second time resulted

in a dramatically increased cytotoxicity in 10T1/2 cells (30-50% loss of viable cells) and significantly diminished GFP expression.

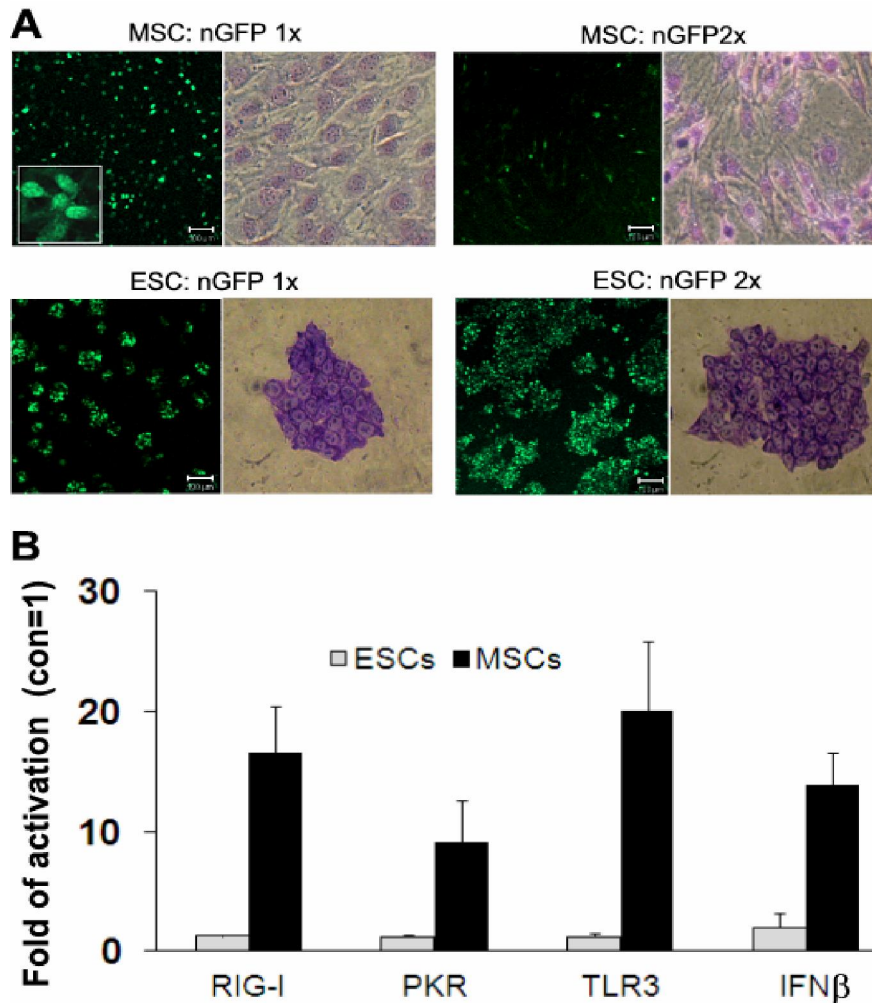


Figure 19. Base-substitution of synthetic nGFP mRNA reduces but not eliminates its immunogenicity. The cells were transfected with a synthetic nGFP-mRNA (nGFP, 300 ng/ml) that has been modified by base modifications. **A**, The transfection was performed twice (1x & 2x) with a 48 h interval. The expression of GFP was examined with a LSM confocal fluorescence microscope at 24 h after each transfection. The boxed area (10T1/2:nGFP 1x) shows enlarged cells to illustrate the nuclear location of GFP. The morphology of transfected cells was examined under a phase contrast microscope after TB staining (400x). **B**, The mRNA levels of IFN β , RIG-I, PKR and TLR3 were determined by RT-qPCR in the cells that were transfected second transfection for 12 h. The results are means \pm SD of three independent experiments.

On the contrary, the second transfection substantially increased GFP expression in D3 cells without causing cell death (Figure 19A, 2x), similar to the results obtained from

EGFP mRNA synthesized in our laboratory without base modifications (Figure 19). It is noted that the first transfection with nGFP-mRNA did not induce IFN β expression in 10T1/2 cells or in D3 cells (data not shown), but the second transfection induced the expression of IFN β and RNA receptors in 10T1/2 cells (10-20 fold) while the effects in D3 cells were negligible (Figure 19B). The results from 10T1/2 cells suggested that base modifications of synthetic mRNA can reduce, but cannot eliminate the immunogenicity.

Discussion

After confirming the deficiency of mESCs in expressing type I IFN when exposed to viral infection and dsRNA viral analogs, it is interesting to investigate their responses to different formats of synthetic ssRNA as viral ssRNA mimics. Short ssRNA induced antiviral responses in highly differentiated epithelial cells (SKOV3 cells) in a manner that depends on the length and the presence of 5'-3p group. Even less differentiated fibroblasts with mesenchymal stem cell properties (10T1/2 cells) displayed strong antiviral responses to 3p-ssRNA and mRNA. These results are consistent with features of ssRNA characterized in other types of somatic cells (Hornung et al., 2006). None of the cellular effects associated with antiviral responses were observed in mESCs. Together with the results previously obtained from viral infection and dsRNA (Wang et al., 2013), the current study provided additional evidence to a general conclusion that mESCs have underdeveloped antiviral mechanisms.

RNA-induced type I IFN expression is mediated by toll-like receptors 3 and 7 localized on the cell surface or on the endosomal membrane and by RIG-I and MDA5

localized in the cytosol (Kawai & Akira, 2011; Stetson & Medzhitov, 2006). Apparently, none of these receptors are functional in mESCs with respect to IFN induction based on this study's results with live viral infection, dsRNA (Wang et al., 2013) and ssRNA (this chapter). Although the detailed molecular mechanisms remain to be elucidated, the low level expression of RNA receptors and their signaling components in ESCs could be at least partly responsible for the deficient antiviral responses (Chen et al., 2010; Wang et al., 2013). RIG-I is the major receptor that mediates the effects of viral ssRNA and synthetic ssRNA, especially those with a 3p-group (Hornung et al., 2006; Kim et al., 2004; Pichlmair et al., 2006; Schlee et al., 2009b). In addition to its well-defined function in inducing type I IFN, a recent study showed that RIG-I activation leads to apoptosis by a mechanism that is independent of IFN expression in SKOV3 cells (Besch et al., 2009). Therefore, the RIG-I pathway may play a major role in mediating the IFN β expression and cytotoxicity induced by ssRNA, as demonstrated in 10T1/2 cells where 3p-80nt-ssRNA induced IFN β expression and cell death within a similar time course. Apparently, the antiviral responses elicited by ssRNA through the RIG-I pathway typically seen in differentiated cells are not induced in ESCs. The only pathway that is functional in mESCs seems to be the PKR pathway, which is activated by polyIC and responsible for polyIC-induced inhibition of translation and cell proliferation as the author previously reported (Wang et al., 2013). Although best characterized as dsRNA-activated kinase, recent studies have indicated PKR can be also activated by ssRNA depending on certain structural features of ssRNA and that there is a synergistic

involvement of RIG-I and IFN (Nallagatla et al., 2011; Nallagatla et al., 2007). The failure of ssRNA to activate PKR in mESCs can be explained by the inactive status of the RIG-I pathway and defected IFN expression mechanism in these cells.

The expression of type I IFN in response to viral infection is the central part of the innate immunity developed in most types of somatic cells. The lack of this mechanism to viral infection and the lack of inflammatory responses to bacterial challenge in ESCs indicated that “innate immunity” is, in fact, not developed in ESCs and has to be acquired during or after their differentiation to other cell types (Yu, Rossi, Hale, Goulding, & Dougan, 2009). While these findings opened up an important area in ESC research for understanding the development of innate immunity during embryogenesis, it is particularly exciting that the underdeveloped antiviral responses in ESCs may provide a “natural solution” to resolve the most challenging issue in application of mRNA-mediated gene expression. As discussed earlier, the problems associated with antiviral responses induced by synthetic mRNA are not insurmountable, but the need of complex modifications is obviously a major barrier for their application. These data demonstrated that 5' capping and 3' polyadenylation of mRNA is not sufficient to repress the immunogenicity of long ssRNA since a single transfection of 10T1/2 cells with m⁷GpppA-EGFP-polyA (without base modifications) caused significant cell death. Base modification of mRNA reduces the immunogenicity since transfection of 10T1/2 cells with nGFP-mRNA (with base modifications) resulted in GFP expression without causing serious cytotoxicity. Therefore, the difference in immunogenicity between the two

GFP-mRNAs is likely due to base modifications. However, it is interesting to note that the effect of base modifications can reduce, but not eliminate the immunogenicity of the mRNA since the effect is limited to a single transfection and the subsequent transfection resulted in apparent antiviral responses in 10T1/2 cells. These findings can be explained by a well-documented phenomenon in antiviral responses; through a positive feedback loop, an initial weak stimulus (the first mRNA transfection in our case) can upregulate the antiviral signaling pathways, thereby “priming” the cells to mount stronger antiviral responses to the subsequent stimulus (second mRNA transfection) (Huang et al., 2006; Matsumoto & Seya, 2008; Pan et al., 2011).

Therefore, immunogenicity intrinsically associated with mRNA (long ssRNA, sequence independent) can sensitize the host cells that have active antiviral mechanisms to repeated mRNA transfection. Without other interventions, such as the knockdown of RNA receptors in the host cells or using inhibitors of IFN signaling pathway, base modification of mRNA alone is not sufficient for successful cell reprogramming that needs multiple mRNA transfections (Angel & Yanik, 2010; Warren et al., 2010). On the other hand, mESCs can be repeatedly transfected even with unmodified 3p-mRNA (ssRNA with the most potent immunogenicity) and can effectively translate functional proteins from the synthetic mRNA either with or without nucleotide modifications.

The ultimate goal of ESC research is to generate clinically usable ESC-derived cells for regenerative medicine. It is increasingly clear that obtaining ESC-derived cells with clinical quality by existing methods is a difficult task. Based on the same principle

and methodology for iPSC reprogramming, a recent study demonstrated that the forced expression of three transfection factors (Etv2, Fli1 and Erg1) that control vascular differentiation effectively converted hESCs to endothelial cells with significantly increased yield and maturity (De Val & Black, 2009; Ginsberg et al., 2012). This study demonstrates that intervention at the transcriptional level can provide a strong internal driving force for cell specific differentiation. However, the expression of the aforementioned transcription factors in that study is also mediated by viral vectors (Ginsberg et al., 2012). Using synthetic mRNA as an alternative gene expression approach may avoid the safety concerns associated with viral vectors. It should be pointed out that mRNA-mediated gene expression described in this study was carried out in undifferentiated mouse ESCs. Generating specific cell types from ESCs must be performed under differentiation conditions and other specific requirements. For example, generating mature and functional endothelial cells from ESCs requires a complex protocol that needs simultaneous expression of Etv2, Fli1, and Erg1 and in combination with repression of TGF β signaling (Ginsberg et al., 2012). The effort toward that goal is underway in our laboratories.

In conclusion this and the previous chapters demonstrate that mESCs have underdeveloped antiviral mechanisms. This finding represents a unique and previously uncharacterized property of mESCs that is not only important for understanding innate immunity development, but can also be exploited for developing mRNA-based gene expression methods that may transform the existing differentiation paradigms.

CHAPTER V

ANTIVIRAL RESPONSES IN MOUSE EMBRYONIC STEM CELLS: DIFFERENTIAL DEVELOPMENT OF CELLULAR MECHANISMS IN TYPE I INTERFERON PRODUCTION AND RESPONSE

Introduction

Type I IFNs, including IFN α , IFN β , IFN ϵ , and IFN ω , are synthesized and secreted by most virally infected somatic cells, while type II IFNs are mainly produced by certain immune cells (Samuel, 2001). After being secreted, type I IFN family members bind the same cell surface receptor complexes and transduce signaling through the same signaling pathway.

Type I IFN signaling pathway

IFN α/β receptors transduce signals through receptor-associated Janus family of tyrosine kinases (JAK) (Figure 20). Jak1 (conjugated with IFNAR2) and Tyk2 (associated with IFNAR1) are activated by the binding of type I IFNs to receptors (Mogensen, Lewerenz, Reboul, Lutfalla, & Uze, 1999). Then activation of Jak1 and Tyk2 leads to phosphorylation of the signal transducer and activator of transcription (STAT) family proteins Stat1 and Stat2. Phosphorylated Stat1 and Stat2 form heterodimers and associate with another protein belonging to the IFN regulatory factor family IRF9 (p48), resulting in the formation of the interferon stimulated gene factor 3 (ISGF-3), which then translocates into the nucleus to trigger downstream gene expression through binding to the interferon stimulated response elements (ISREs) (Darnell, 1997; Stark, Kerr, Williams,

Silverman, & Schreiber, 1998). ISREs are cis-acting DNA elements responsible for the type I IFN-induced gene expression with the consensus sequence AGTTTCNNTTTCNPy, which is the binding site for ISGF-3 and some IRFs (Darnell, 1997; Leonard & O'Shea, 1998).

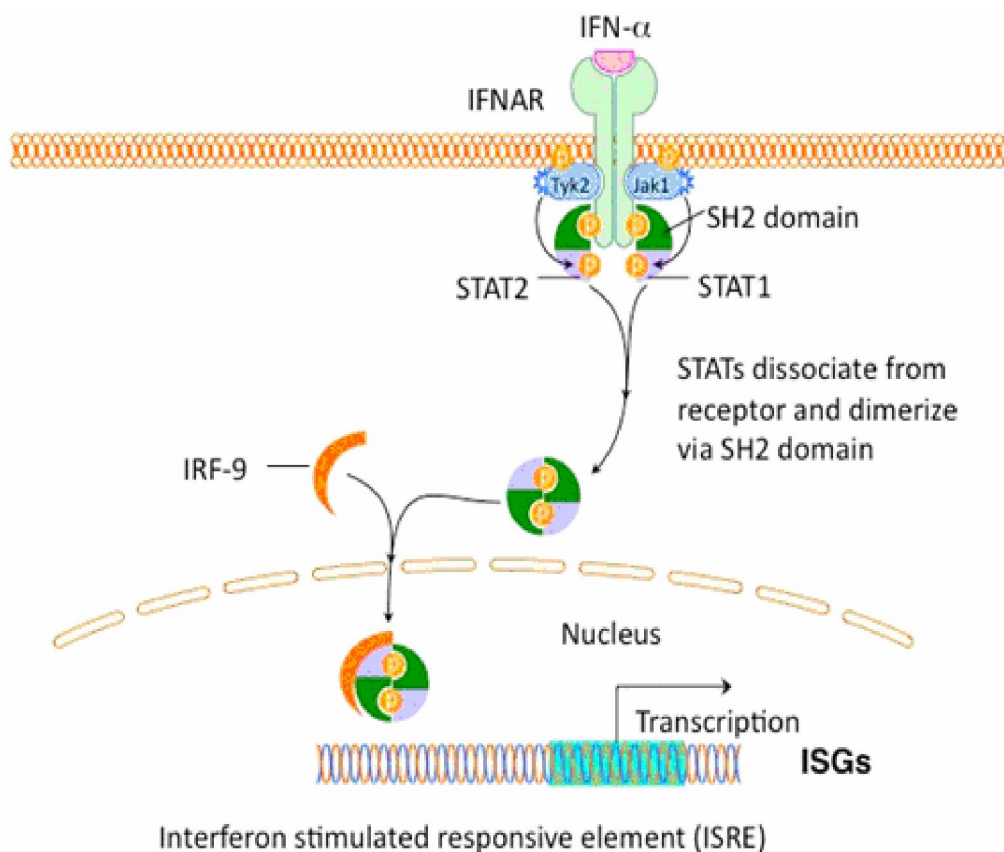


Figure 20. Type I IFN downstream signaling. JAK family kinases conjugated with IFNAR are activated through type I IFN binding to receptors and promote phosphorylation of STAT1 and STAT2, which form a complex with IRF-9 to induce expression of ISG. (Masumi, 2013)

The cell surface receptor of type I IFN consists of 2 subunits: IFNAR1 and IFNAR2. Upon binding type I IFN, 2 subunits form a heterodimer (Samuel, 2001). IFNα/β receptors play essential roles in anti-viral immunity as evidenced by the failure of IFNα/β null mice to establish an anti-viral state.

Several negative regulators exist in cells to control the amplitude and duration of type I IFN signaling. Proteins in the family of the suppressor of cytokine signaling (SOCS) cast negative regulation on the JAK-STAT pathway (Naka, Fujimoto, & Kishimoto, 1999; Starr et al., 1997). They all have two characteristic conserved domains, a central SH2 domain and a SOCS box at the C-end (Naka et al., 1999; Starr et al., 1997). SOCS proteins which are expressed at low basal levels can be induced by IFN signaling and form a negative feedback loop, inhibiting IFN signaling by suppressing JAK kinase activation (Endo et al., 1997). SOCS1 is an important regulator of innate immunity that efficiently inhibits STAT1 phosphorylation (Alexander et al., 1999; Brysha et al., 2001). In addition to SOCS family members, protein inhibitor of activated stat-1 (PIAS-1) can also suppress IFN signaling by directly binding to Stat1, blocking its DNA-binding activity (Liao, Fu, & Shuai, 2000; Liu et al., 2004). Another way to counteract IFN signaling is to dephosphorylate target molecules. SH-2 containing phosphatase-1 (SHP-1) directly interacts with and dephosphorylates JAKs to suppress various cytokine signaling (David, Chen, Goelz, Lerner, & Neel, 1995).

IFN-induced effectors

Type I IFNs can suppress a wide range of viral replication in vitro and in vivo. Their effects are mediated by more than 200 downstream genes induced by IFN signaling, including double-stranded RNA dependent protein kinase (PKR), 2',5'-oligoadenylate synthetase (OAS), the RNA-specific adenosine deaminase (ADAR), and the Mx protein GTPase (Samuel, 2001).

PKR

PKR is an IFN-induced, RNA-dependent protein kinase with multiple substrates (Clemens, 1997). While PKR is mainly found in the cytoplasm and associated with ribosomes, some amounts of PKR localize to the nucleus after IFN treatment (Jimenez-Garcia, Green, Mathews, & Spector, 1993; Samuel, 1993; Thomis, Doohan, & Samuel, 1992). After being induced and activated, PKR phosphorylates various substrates to mediate different biological consequences.

Antiviral Actions of Interferon

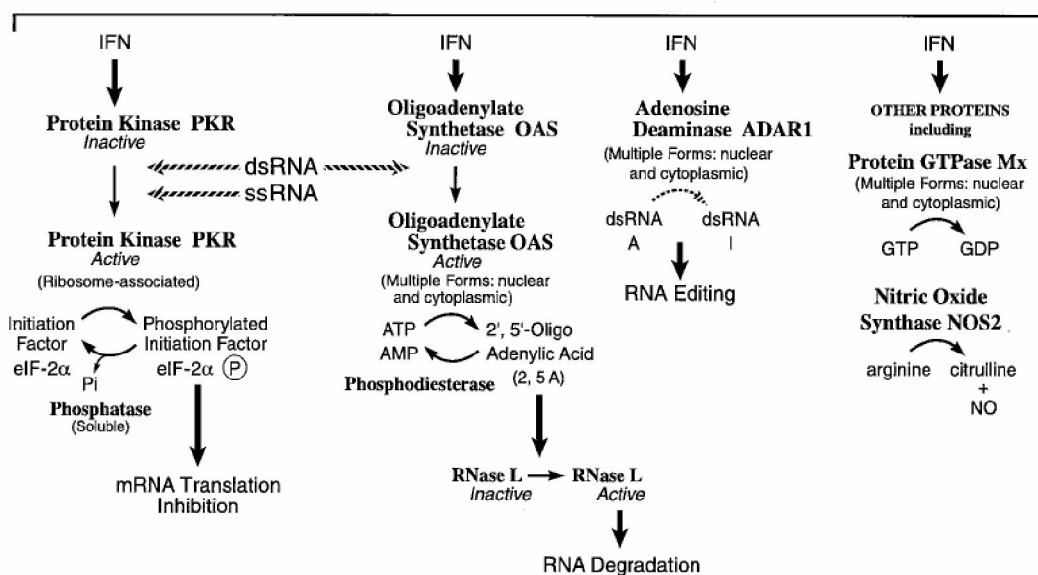


Figure 21. Functions of some IFN-induced proteins. PKR inhibits translation of viral and cellular RNA by phosphorylating eIF-2α; 2',5'-oligoadenylic acid, which is synthesized through OAS catalysis, activates RNase L to degrade viral and cellular RNA; ADAR1 converts adenosine in the cellular and viral RNA to inosine to affect their function and stability; Mx family GTPases interfere with the viral replication directly through targeting viral nucleocapsids and RNA synthesis. (Samuel, 2001)

Translation control and eIF2α: In mammals, eIF2 (consisting of α, β and γ)

subunits binds Met-tRNA in a GTP-dependent manner to promote its delivery to the 40S ribosome subunit (Hershey, 1991; Majumdar & Maitra, 2005). Once Met-tRNA is

delivered, eIF2 dissociates from the 48S initiation complex via eIF5-dependent GTP hydrolysis (Hershey, 1991; Majumdar & Maitra, 2005). Active eIF2-GTP complexes are continuously regenerated by GDP-GTP exchange catalyzed by eIF2B. After phosphorylation on Ser51 of eIF2 α , affinity of eIF2 to eIF2B increases for 100-fold, leading to the inhibition of eIF2B and translation initiation (Sudhakar et al., 2000). Given that the concentration of eIF2B is low compared with eIF2, a small increase in eIF2 α phosphorylation will cause an exacerbated effect on protein synthesis (Hershey, 1991).

Phosphorylation of eIF2 α serves not only as a control on protein synthesis but also as response to stress conditions. Four kinases (PKR, GCN2, PERK, and HRI) that can exert this modification sense stress signals and regulate cell responses (Dever, 2002).

Although translation of most mRNAs is inhibited by eIF2 α phosphorylation, few mRNAs involved in stress response get enhanced translation. These genes include yeast GCN4 and mammalian activating transcription factor 4 (ATF-4), ATF-3, and CAT-1 mRNAs (Dever et al., 1992; Guerra, Lopez-Fernandez, Garcia, Zaballos, & Esteban, 2006; Lu, Harding, & Ron, 2004; Yaman et al., 2003). Translation of these mRNAs is inhibited by the upstream short open reading frame (ORF) for short peptides in normal condition, while a reduced number of active initiation complex promotes re-initiation at bona fide GCN4 and ATF-4 ORFs to give functional proteins under stress conditions because eIF2 α phosphorylation limits the number of active 43S complexes (Dever, 2002). Moreover, some viral mRNAs can make use of a stable stem-loop structure downstream of the AUG codon that stalls the ribosomes on the correct site for translation initiation,

while others can use eIF2A instead of eIF2 to deliver the Met-tRNA_i and initiate translation under high level of eIF2 α phosphorylation (Ventoso et al., 2006; Wilson, Pestova, Hellen, & Sarnow, 2000).

PKR was first related with its effects on protein synthesis and its substrate eIF2 α . PKR is one of 4 kinases that phosphorylate eIF2 α (Barber, 2001; Dever, 2002; Harding et al., 2000; Williams, 1999, p. 6112). Phosphorylation on serine51 of eIF2 α prevents the recycling of this factor required for ongoing translation, causing general translational inhibition (Garcia et al., 2006). So accumulated dsRNAs activate PKR, triggering phosphorylation of eIF2 α and inhibiting translation of viral mRNA (Balachandran et al., 2000; Lee, Bablanian, & Esteban, 1996; Lee et al., 1993; Stojdl et al., 2000). It has been shown that phosphorylation on Thr446 is essential for substrate recognition and modification (Su et al., 2006). Furthermore, as a translation regulator, PKR was found to associate with ribosomes, mainly 40S subunits (Kumar, Srivastava, & Kaufman, 1999; Zhu, Romano, & Wek, 1997). The PKR localization in ribosomes explains the local activation pattern of PKR in response to limited stimuli (Balachandran et al., 2000; Ben-Asouli et al., 2002; Kaufman, Davies, Pathak, & Hershey, 1989; Zhu et al., 1997). This localization and activation pattern may guarantee specificity and efficiency of PKR.

IRF-1: PKR was reported to mediate the growth inhibitory activity of IRF-1 (Kirchhoff et al., 1995). On the other hand, IRF-1 may mediate PKR-triggered apoptosis (Der, Yang, Weissmann, & Williams, 1997). Supporting this view, the activation of IRF-1 in response to IFN γ and polyIC is defective in PKR null mice (Kumar et al., 1997).

STAT: PKR associates with STAT1 in a kinase-independent manner and may inhibit the DNA binding activity of STAT1 (Wong et al., 1997). PKR can also regulate phosphorylation of STAT1 indirectly through the ERK-MAPK pathway (Ramana et al., 2000). As mentioned above, PKR is required for full activation of STAT3 and downstream gene induction in response to PDGF (Deb, Zamanian-Daryoush, Xu, Kadereit, & Williams, 2001b).

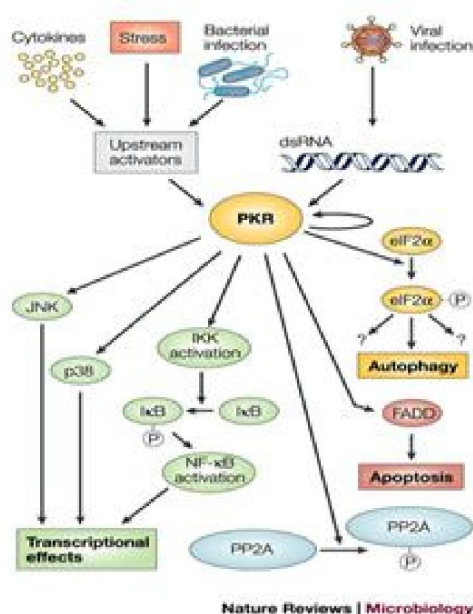


Figure 22. Substrates and biological effects of PKR. PKR, which can be activated by double-stranded RNA and cell stresses, phosphorylates a broad range of substrates including eIF2 α , NF- κ B, JNK, and p38, leading to diverse biological consequences. (Kirkegaard, Taylor, & Jackson, 2004)

Studies have shown that cells or mice deficient in PKR exhibit impaired responses to different TLR ligands, implying the involvement of PKR in TLR signaling (Goh, deVeer, & Williams, 2000). Supporting this view, PKR has been shown to interact with TIRAP and is phosphorylated in LPS-stimulated macrophages (Horng et al., 2001). PKR is also activated in TLR9 signal transduction in response to CpG (Horng et al., 2001).

Moreover, PKR is also engaged in dsRNA-activated TLR3 signaling via recruitment to TAK1-containing complex (Jiang et al., 2003).

PKR is phosphorylated and activates expression of immediate early gene c-fos in response to platelet-derived growth factor (Deb et al., 2001b; Mundschau & Faller, 1995). After being activated by PDGF, PKR plays important roles in phosphorylating STAT3 on Serine727 (Deb et al., 2001b). Also, PKR null MEF shows deficient p38 activation in response to IL-1 (Goh et al., 2000).

PKR serves as an activator of signaling in stress activated protein kinases and mediates activation of JNK and p38MAPK (Chu et al., 1999; Goh et al., 2000). PKR is required for the full activation of JNK and p38 in response to LPS and cytokines (Goh et al., 2000). Using PKR knockout MEF, PKR has been shown to be required for p38 MAPK activation in response to dsRNA, LPS, and proinflammatory cytokines but not in response to other forms of stress (Goh et al., 2000).

Biological consequences of PKR activation are diverse, affecting cell proliferation, cell death, and differentiation. Most effects of PKR activation can be explained by the signaling pathways in which it is involved. For example, apoptotic induction can be related with phosphorylation of eIF2 α , NF- κ B, p53, and ATF-3 (Garcia et al., 2006).

PKR has been implicated in cycle regulation. However, its effects seem to be complex and context-dependent because PKR activity is widely related with various signaling pathways. Aside from possible involvement of p53 or NF- κ B, phosphorylation of eIF2 α has been shown to cause delayed G1-S transition. PKR and PERK induce

proteasome-dependent degradation of cyclin D1 through eIF2 α phosphorylation, leading to G1 arrest (Raven et al., 2008). Also, these two kinases promote degradation of p53 via GSK3 activation (Baltzis et al., 2007). On the other hand, GCN2, an eIF2 α kinase, inhibits loading of components of pre-replication complex (pre-RC) to induce a novel G1 checkpoint under UV irradiation in yeast (Tvegard et al., 2007).

OAS and RnaseL

IFN signaling promotes the degradation of viral and cellular RNA and involves two enzymes: 2',5'-oligoadenylate synthetase (OAS) and Rnase L. In the first step, OAS catalyzes the synthesis of oligoadenylates through using ATP in a 2'-specific nucleotidyl transfer reaction (Kerr & Brown, 1978). Then, in the second step, a latent endoribonuclease, Rnase L, is activated through binding to newly synthesized 2-5 A oligonucleotides.

Three forms of OAS (OAS1, OAS2, and OAS3) have been identified in human cells (Reubinoff et al., 2000). Oligomerization of OAS1 and OAS2, which form tetramers and dimers, respectively, is necessary for their activation (Ghosh, Sarkar, Guo, Bandyopadhyay, & Sen, 1997; Sarkar, Ghosh, Wang, Sung, & Sen, 1999). Like PKR, OAS is activated by dsRNA and possesses separate subdomains responsible for RNA-binding activity and enzymatic activity (Reubinoff et al., 2000; Sarkar & Sen, 1998). However, there is no obvious homology in the dsRNA-binding regions of OAS proteins and PKR (Ghosh et al., 1991). OAS enzymes are activated during viral infection, although in most conditions the exact RNA activators have not been well-defined

(Reubinoff et al., 2000). The induction of OAS by IFN varies in different cell types and growth state. For example, 10-fold increase was found in Hela cells with a high basal enzyme level while OAS can be induced by 10,000 fold in chicken embryonic cells which have a low basal level (Baglioni, Maroney, & West, 1979; Ball, 1979). All three forms of OAS can be induced by IFN α , IFN β , and IFN γ (Chebath, Benech, Hovanessian, Galabru, & Revel, 1987).

Rnase L, normally residing in the cells as latent and inactive monomers, requires the presence of functional 2-5A oligomers to form stable homodimers and acquire endoribonuclease activity (Dong & Silverman, 1995; Dong et al., 1994; Hassel, Zhou, Sotomayor, Maran, & Silverman, 1993). The ability of 2-5A to mediate dimer formation of Rnase L correlates with its ability of activation (Dong & Silverman, 1995). After being activated, Rnase L promotes the degradation of both viral and cellular RNAs, including rRNA, through cleaving the UpXp sequences on the 3' side (Floyd-Smith, Slattery, & Lengyel, 1981; Wreschner, McCauley, Skehel, & Kerr, 1981). Rnase L is constitutively expressed and present in cells, although Northern blot showed that type I IFN increases the steady-state amount of Rnase L mRNA by three folds in mouse cells (Floyd-Smith & Denton, 1988; Zhou, Hassel, & Silverman, 1993). Knocking out Rnase L genes effectively produces a phenotype of 2-5A knockout model, since the only function of 2-5A is to activate latent Rnase L (Samuel, 2001). Rnase L knockout mice die more rapidly than the wild type control under the infection with EMC viruses (Zhou et al., 1997; Zhou, Paranjape, Der, Williams, & Silverman, 1999). Also, deficiency in apoptosis

has been reported in Rnase L knockout MEF (Zhou et al., 1997). IFN-induced transcripts may be further elevated in cells without functional Rnase L. The 2-5A system may function to attenuate and limit IFN response by destabilizing IFN-induced mRNAs, a hypothesis supported by kinetic analysis of Rnase L knockout and wildtype MEF (Li et al., 2000).

Other IFN regulated genes

Gene expression and function can be regulated and altered at the post-transcriptional level by RNA modification. For example, the deamination of adenosine to yield inosine provides an efficient way to control or alter functions of viral and cellular RNA (Bass, 1997; Cattaneo, 1994). Double-stranded RNA-specific adenosine deaminase (adenosine deaminase acting on RNA, ADAR1), one important RNA editing enzyme, is a downstream target of IFN (George & Samuel, 1999; Patterson, Thomis, Hans, & Samuel, 1995; Patterson & Samuel, 1995). A-to-I transitions catalyzed by ADAR destabilize the dsRNA helix to the favor single stranded character because the newly generated IU pair is unstable compared with the original AU base pairs (Bass & Weintraub, 1988). Moreover, the translation of modified RNA can be affected since hypoxanthine, which is the base of inosine, is typically recognized as guanine by translational machinery (Alberts et al., 1994).

Protein MxA (human) and Mx1 (mouse) of the MX family proteins have been well-characterized in IFN-induced anti-viral activity, evidenced by the finding that Mx alone is enough to block replication of virus in the absence of any other type I IFN

downstream genes (Arnheiter, Frese, Kambadur, Meier, & Haller, 1996; Haller, Frese, & Kochs, 1998). The anti-viral activity depends on their intrinsic GTPase activity (Pitossi et al., 1993). Mx proteins are induced only by type I IFN but not IFN γ or other cytokines (Arnheiter et al., 1996; Simon, F  h, Haller, & Staeheli, 1991). The mechanisms and spectrum of antiviral activity of Mx proteins depend on their subcellular localization and the type of infection.

Correlating with their subcellular localization, Mx1 blocks the multiplication of influenza virus mainly at the step of transcription catalyzed by the virion-associated polymerase, while the human MxA interacts with the viral nucleocapsid by binding to the NP nucleoprotein component (Kochs & Haller, 1999; Krug, Shaw, Broni, Shapiro, & Haller, 1985; Pavlovic, Haller, & Staeheli, 1992).

Interferon stimulated gene 15 (ISG15) is a ubiquitin-like protein induced by IFNs (Morales & Lenschow, 2013). ISG15 forms conjugates with a large population of target proteins through the process called ISGylation which involves E1 activating enzymes, E2 conjugating enzymes and E3 ligases. Many important ISGs have been identified as ISG15 targets. ISGylation of IRF3 stabilizes activated IRF3, thus helping to maintain type I IFN signaling (Lu et al., 2006; Shi et al., 2010). RIG-I was also found to be ISGylated and thus down-regulated in protein levels, dampening the IFN responses (Kim, Hwang, Imaizumi, & Yoo, 2008). PKR was found to be activated in the absence of RNA in a ISG15-dependent manner as supported by the fact that such RNA-independent activation cannot be induced in a non-ISGylatable PKR (Okumura et al., 2013). Other

than host proteins, viral proteins like NS1 protein of influenza A virus and capsid protein of HPV were shown to be ISGylated, leading to reduced replication or infectivity (Durfee, Lyon, Seo, & Huibregtse, 2010; Tang et al., 2010; Zhao, Hsiang, Kuo, & Krug, 2010). Moreover, unconjugated ISG15 secreted to extracellular space protected mice from CHIKV induced lethality through regulating production of cytokines and chemokines (Werneke et al., 2011).

The above-mentioned genes are the most well-established IFN-induced anti-viral effectors in a single cell model. Some other proteins like major histocompatibility complex proteins are involved in multiple-cell interactions (Samuel, 2001). More than 100 out of 6800 human genes were identified to be differentially regulated by IFN treatment of human fibrosarcoma cell line (Der, Zhou, Williams, & Silverman, 1998). A IFN response is a broad-reaching reaction with a genomic change of gene expression.

Methods

Cell culture

D3 and DBA252 mESCs were maintained in the standard mESC medium as previously described (Wang et al., 2013). C3H10T1/2 (10T1/2, a line of mouse embryonic fibroblasts, ATCC) were cultured in DMEM that contains 10% fetal calf serum and 100 units/ml penicillin and 100 µg/ml streptomycin. All cells were maintained at 37 °C in a humidified incubator with 5% CO₂. Most experiments were performed with D3 cells, and key results were confirmed with DBA252 cells.

Preparation of virus stocks and titer determination

La Crosse virus (LACV, SM6 v3), West Nile virus (WNV, strain CT 2741) and Chikungunya virus (CHIKV, LR 2006 OPY 1 strain) were propagated in Vero cells (African green monkey kidney cell line, ATCC). Titers of virus stocks were determined by plaque assay as previously described (Bai et al., 2005).

Cell treatment

mESCs and 10T1/2 cells were plated at ~40% and ~70% confluence, respectively and cultured for ~24 h before experiments. The conditions for cell infection with different viruses were specified in individual experiments. The cellular responses to type-I IFNs were determined with mouse recombinant IFN α (IFN α -2, 1×10^8 U/mg, eBioscience) and human recombinant IFN β or IFN ω (5×10^8 U/mg, 1×10^8 U/mg, respectively, Peprotech) that are active on mouse cells. The effects of IFNs on viral replication were determined by viral titers in the media of infected cells. For polyIC (polyinosinic:polycytidylic acid, a synthetic dsRNA) treatment, the cells were transfected with polyIC using a DharmaFECT reagent (Thermo Scientific). The control cells were transfected with the DharmaFECT reagent alone.

Immunodetection of STAT1 nuclear translocation

Immunostaining was performed according to our published method (Wang & Guo, 2012). Briefly, treated cells were fixed with 4% paraformaldehyde. They were then incubated with antibodies that recognize phosphorylated STAT1 (pSTAT1, Cell Signaling Technology) followed by an incubation with rhodamine-conjugated secondary antibodies.

The cells were then analyzed under a LSM 510 laser-scanning confocal microscope (Zeiss).

siRNA transfection

siRNA targeting SOCS1 (Santa Cruz Biotechnology) were transfected to the cells with the DharmaFECT reagent as previously described (Wang et al., 2013). The cells were then analyzed for knockdown efficiency and for mRNA levels of SOCS1.

Reverse transcription Real-time quantitative polymerase chain reaction (RT-qPCR), cell proliferation, viability, and cell cycle analysis, protein analysis by flow cytometry, and Western blot, and statistical analysis were described in the Method part of Chapter III.

Results

IFN β and IFN ω Protect mESCs from LACV Induced Cell Death

Antiviral activity is considered the most important physiological function of type I IFNs. Previous data have shown that mESCs and 10T1/2 cells (a mouse embryonic fibroblast cell line) are susceptible to LACV-induced lytic cell death (Wang et al., 2013). Therefore, these cells were used to analyze the antiviral effects of IFN β and IFN ω , a best studied and a less characterized type I IFNs, respectively.

As shown in Figure 23A, LACV infection caused about 50% cell death in 10T1/2 cells, which was attenuated in the cells that were pretreated with IFN β or IFN ω . Similarly, IFN β pretreatment of mESCs (D3) protected the cells from subsequent LACV-induced cell death in a dose-dependent manner (Figure 23B). Similar effects were observed with

IFN ω (data not shown). These results suggest that mESCs can mediate the antiviral effects of type I IFNs like differentiated fibroblasts.

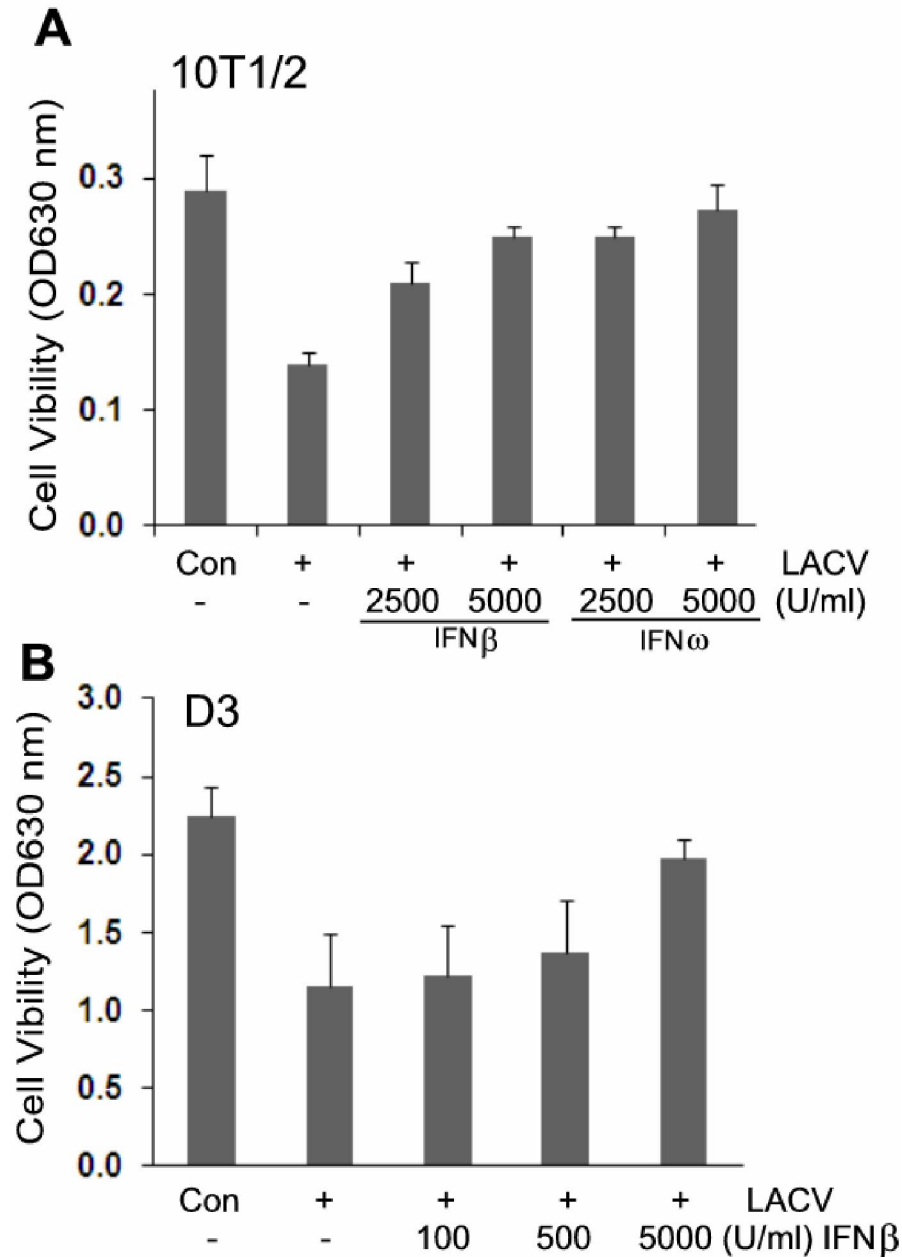


Figure 23. IFN β and IFN ω protect 10T 1/2 cells and mESCs from LACV induced cell death. A, 10T1/2 cells were pretreated with or without IFN β or IFN ω for 24 h. They were then infected with LACV (m.o.i.=1). B, D3 cells were pretreated with different concentrations of IFN β for 24 h. The cells were then were infected with LACV (m.o.i.=10). Viable cells in both A and B were determined at 48 h post infection by toluidine blue cell staining. The control (Con) represents cells without viral infection. The

values are means \pm SD of a representative experiment performed in triplicates. The experiments were performed at least twice with similar results.

IFN β Represses Viral Replication in both 10T1/2 Cells and mESCs

To determine the molecular and cellular mechanisms that protect the cells from cytopathicity of LACV infection, the effects of IFN β on LACV replication were analyzed. By analyzing the titer of viruses released to the medium from LACV infected cells, these results showed that the viral load was significantly reduced in both 10T1/2 cells and mESCs that were pretreated with IFN β (Figure 24A). The repression of viral replication by IFN β was further confirmed by reduced expression of a LACV gene that encodes an M-segment protein (Gc protein) (Soldan et al., 2010). As shown in Figure 24B, the expression of Gc protein was detected in a large population of mESCs exposed to LACV. Pretreatment of the cells with IFN β dramatically reduced the number of these cells, paralleling with the reduced viral titer as shown in Figure 24A. Then, the effects of IFN β on LACV-induced expression of IFN α and IFN β were analyzed. In 10T1/2 cells, LACV induced dramatic increase of both IFN α and IFN β . Pretreatment with either IFN β or IFN ω reduced the IFN α and IFN β level by \sim 75% in 10T1/2 cells. On the other hand, mESCs did not express IFN α or IFN β in response to LACV infection (Figure 24C), and pretreatment with either IFN β or IFN ω had no effect on IFN α or IFN β induction.

Therefore, IFN β or IFN ω can protect ESCs from LACV's cytopathic effect, but they do not alter the deficiency of ESCs in expressing type I IFNs (Wang et al., 2013). Additional experiments demonstrate that IFN β also repressed the replication of West Nile Virus (WNV) in mESCs and in 10T1/2 cells. In comparison with LACV (Figure 24A),

the inhibitory effect of IFN β on WNV replication in mESCs is less potent, but the effect is significant and consistent (Figure 24D).

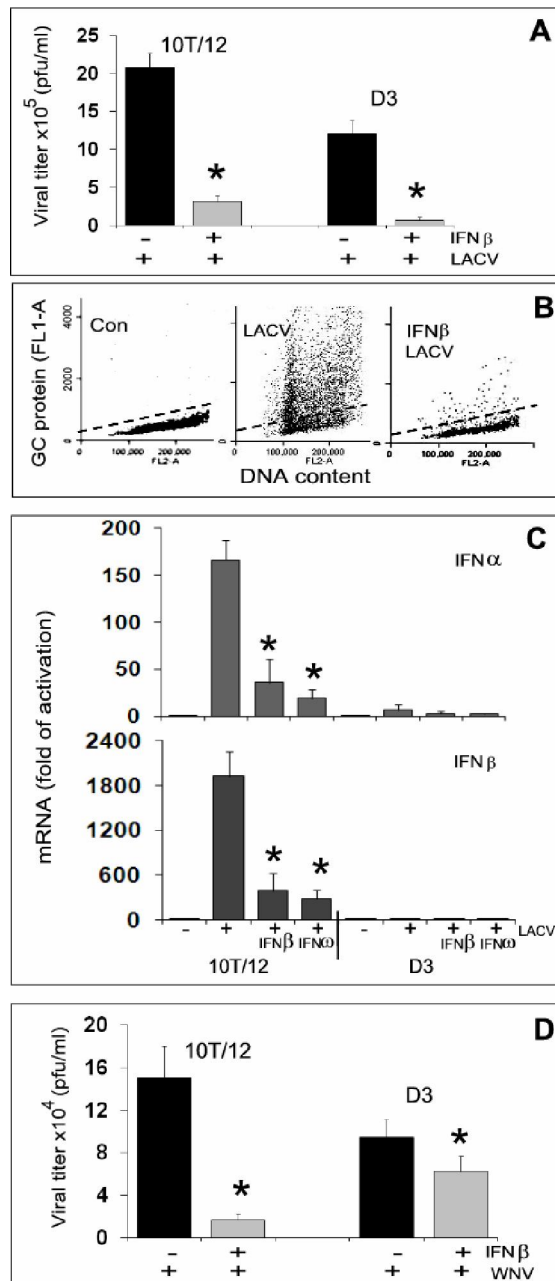


Figure 24. IFN β represses viral replication in 10T1/2 cells and mESCs. 10T1/2 and D 3 cells were pretreated with 5000 U/ml IFN β or IFN ω for 24 h followed by infection with LACV at m.o.i. of 1 and 10, respectively (A-C) or followed by infection with WNV at the m.o.i. of 1 and 5, respectively (D). A, The viral titers in the medium collected at 24 h of infection were determined by plaque assay. The values are means \pm SD of three independent experiments. B, Cells expressing LACV Gc protein (the populations above

the dotted lines) were detected by flow cytometry at 24 h post infection. The control (Con) represents cells without viral infection. C, The induction of IFN α and IFN β mRNA by LACV infections was determined by RT-qPCR at 12 h post infection. The mRNA level in Con is designated as 1. The values are means \pm SD of three independent experiments. D, The viral titers in the medium collected at 24 h post infection were determined by plaque assay. The values are means \pm SD of three independent experiments. The difference is considered to be statistically significant when $p < 0.05$ (*).

Viral Infection Induced Antiviral Molecules in 10T1/2 Cells and mESCs and the Effect of IFN β

In response to viral infection, host cells use various mechanisms to limit viral infectivity. Viral infection-induced ISGs play key roles in host antiviral activities. The author examined three well-characterized ISGs; 2'-5'-oligoadenylate synthetase 1 (OAS1), which activates ribonuclease L (RNase L), thereby hydrolyzing cellular and viral RNA; PKR, which inhibits protein synthesis and host cell proliferation, thereby limiting viral replication; and ISG15, which is a ubiquitin-like protein that leads to the degradation of both host and viral proteins (Garcia et al., 2007; Samuel, 2001; Skaug & Chen, 2010). As shown in Figure 25A, LACV infection caused significant upregulation of *Pkr*, *Oas1a*, and *Isg15* in 10T1/2 cells, which were slightly potentiated by IFN β pretreatment. On the other hand, none of these three genes in mESCs were induced by LACV infection. However, they were induced by several folds when the cells were pretreated with IFN β (Figure 25B).

A logical explanation for the above results is that LACV-induced type I IFNs, via autocrine signaling, were responsible for the expression of *Pkr*, *Oas1a*, and *Isg15* in LACV infected 10T1/2 cells (Figure 25A, LACV) while the induction in LACV-induced cells with IFN pre-treatment is the combined effect of autocrine and exogenously added

IFNs (Figure 25A, IFN β LACV). On the other hand, the expression of the three genes in mESCs was not induced by LACV infection due to the deficiency of these cells in expressing IFNs (Wang et al., 2013) (Figure 25B, LACV), but they can be induced by exogenously added IFN β (Figure 25B, IFN β LACV), indicating that mESCs can detect and respond to IFN β .

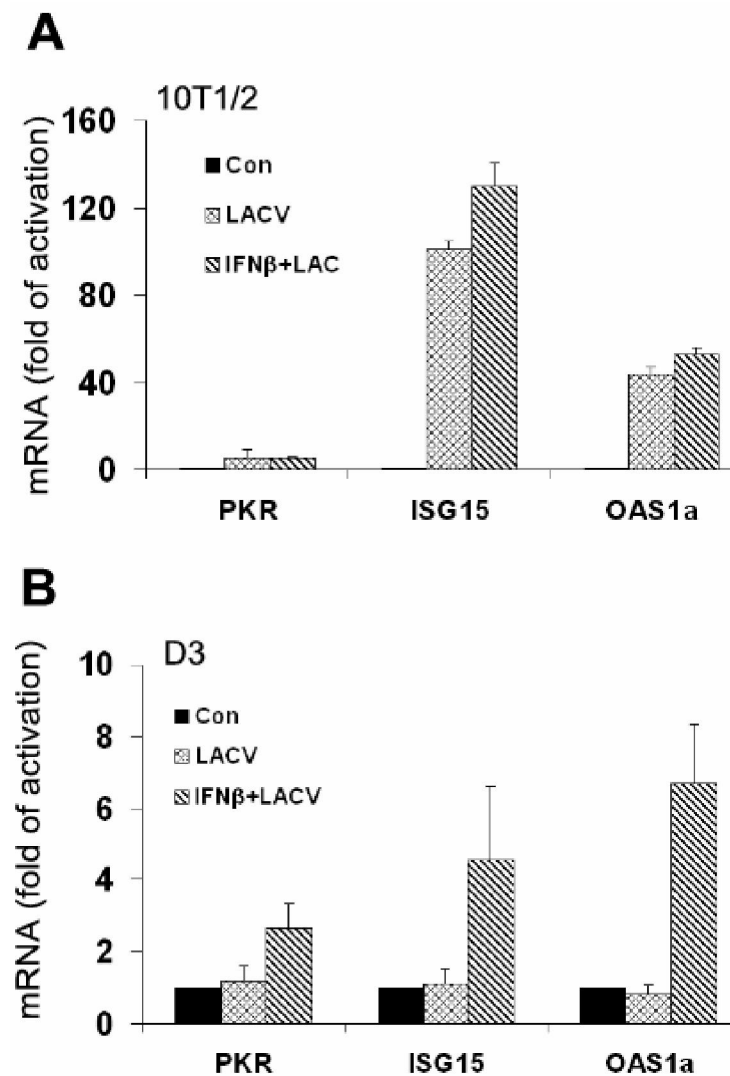


Figure 25. LACV infection induced antiviral molecules and the effects of IFN β . 10T1/2 cells (A) and D3 cells (B) were infected with LACV at m.o.i. of 1 and 10, respectively (LACV), or they were pretreated with 5000 U/ml IFN β for 24 h followed by infection with LACV (IFN β + LACV) for 12 h. The mRNA levels of the PKR, ISG15 and OAS1a were determined by RT-qPCR. The results are expressed as fold-activation where the

mRNA level in control cells (Con, cells without viral infection) is designated as 1. The values in A are means \pm SD of a representative experiment performed in triplicates. The experiments were performed at least twice with similar results. The values in B are means \pm SD of three independent experiments.

IFN β Induced ISG Expression and its Priming Effect in mESCs

To further test the responsiveness of mESCs to IFN β , The author examined the effects of IFN β on the expression of *Pkr*, *Oas1a*, and *Isg15*. As illustrated in Figure 26, IFN β induced the transcription of the three genes in 10T1/2 cells as a positive control (Figure 26A). This effect was observed similarly in two different lines of mESCs (Figure 26B, D3 and DBA) although the magnitude of induction of the three genes in mESCs is lower than in 10T1/2 cells. Further analysis by Western blot showed that IFN β increased PKR protein level in a dose dependant manner in both mESCs and 10T1/2 cells, paralleling with its mRNA induction (Figure 26C).

In differentiated cells, it is known that the pretreatment of cells with type I IFNs enhances their antiviral activities to the subsequent viral infection, a phenomenon known as the IFN priming effect (Samuel, 2001). Our results from LACV infection experiments indicate that the priming mechanism is functional in both 10T1/2 cells and mESCs (Figure 23). The priming effect is partly attributed to the upregulation of viral RNA receptors in addition to the induction of ISGs. To confirm the priming mechanism in mESCs, mESCs were primed with IFN β followed by treatment of polyIC as a viral RNA mimic. As shown in Figure 26D, polyIC alone only slightly induced the expression of *Rig-I* and *Tlr3* (two major receptors for viral RNAs). However, its effect was strongly potentiated in the cells that were pretreated with IFN β , a pattern that fits well with the

IFN priming described in differentiated cells (Stewart, Gosser, & Lockart, 1971). However, priming mESCs with IFN β did not result in the expression of IFN β in response to polyIC (data not shown).

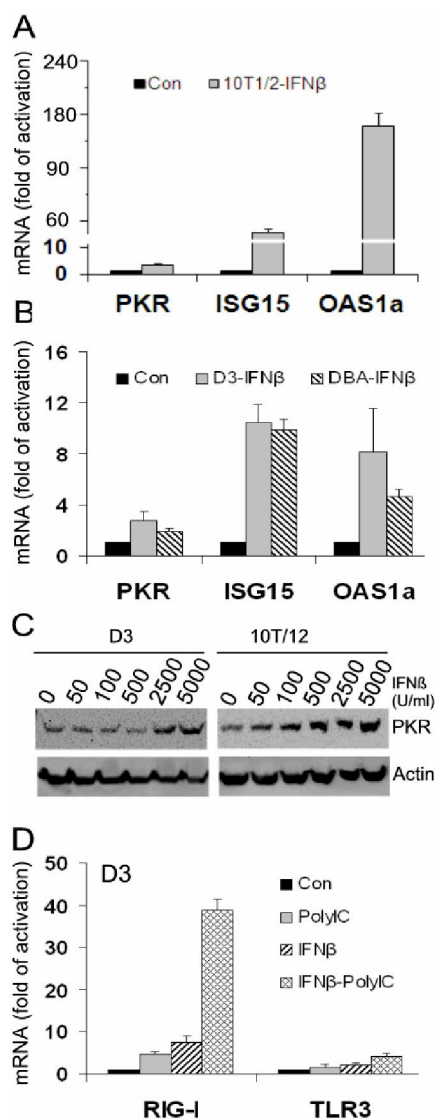


Figure 26. IFN β induces ISG expression and its priming effects. 10T1/2 cells (A) and two different mESC lines (D3 and DBA cells) (B) were treated with 5000 U/ml IFN β for 12 h. The mRNA levels of PKR, ISG15 and OAS1a were determined by RT-qPCR. The results are expressed as fold-activation where the mRNA level in control cells (Con, cells without IFN β treatment) is designated as 1. The values in A are means \pm SD of a representative experiment performed in triplicates. The experiments were performed at least twice with similar results. The values in B are means \pm SD of three independent experiments. C, IFN β -induced PKR was analyzed by Western blot β -actin was used as a

control for protein loading. D, D3 cells were transfected with polyIC (300 ng/ml) or treated with IFN β 5000 U/ml) separately, or pretreated with IFN β 5000 U/ml) for 24 h followed by polyIC transfection. The mRNA levels of PKR, ISG15 and OAS1a were determined by RT-qPCR at 12 h incubation. The results are expressed as fold-activation where the mRNA level in Con is designated as 1. The values are means \pm SD of a representative experiment performed in triplicates. The experiments were performed at least twice with similar results.

The Relative Expression Levels of Type I IFN Signaling Molecules in mESCs and 10T1/2 cells

All of the results thus far demonstrate that the signaling pathways that mediate the effects of type I IFN are functional in mESCs. However, the levels of ISGs induced by IFN β in mESCs are notably lower than in 10T1/2 cells.

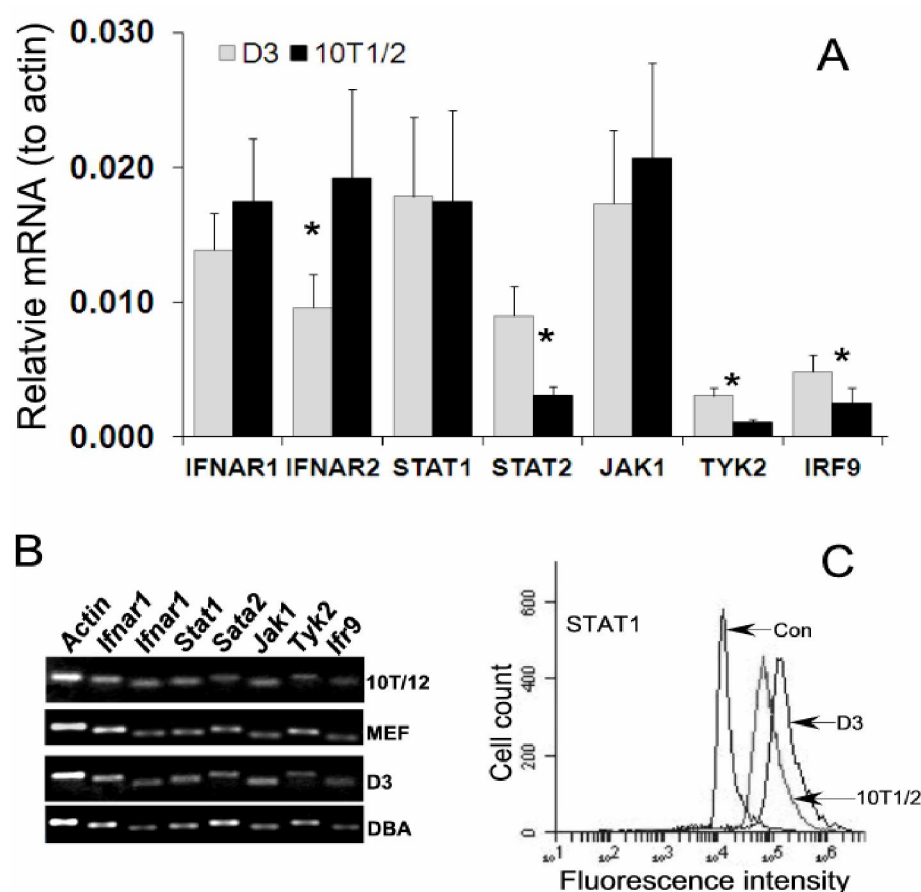


Figure 27. The relative expression levels of type I IFN signaling molecules in mESCs and fibroblasts. **A**, the relative mRNA levels of indicated genes in D3 cells and 10T1/2 cells were determined by RT-qPCR. The relative mRNA level of each gene between the

two cell types was compared after normalization to β -actin mRNA in each cell type. The results are means \pm SD of three independent experiments. The difference is considered to be statistically significant when $p < 0.05$ (*). B, The RT-PCR products (obtained from the reactions before reaching plateau phase) of indicated genes in each cell type were analyzed by agarose gel electrophoresis. The relative abundance of each gene was compared to β -actin in each cell type (i). C, The expression of STAT1 was determined by flow cytometry after the cells were first immunostained with anti-STAT1 antibodies followed incubation with FITC-labeled secondary antibodies. The expression level was proportional to fluorescence intensity. The control (Con) represents cells without STAT1 antibodies.

To determine the reasons for these discrepancies, the basal expression levels of the major signaling molecules in the IFN pathway were analyzed. As shown in Figure 27, quantitative RT-qPCR analysis indicated that the mRNA of *Ifnar1*, *Jak1*, and *Stat1* are expressed at comparable levels in D3 cells and 10T1/2 cells, while the mRNA levels of *Tyk2*, *Stat2*, and *Irf9* are actually higher in D3 cells than in 10T1/2 cells. The only gene with low mRNA expression in D3 cells is *Ifnar2* (Figure 27A). A comparative analysis of two lines of mESCs (D3 and DBA) and two types of fibroblasts (MEF and 10T1/2) showed that the mRNA of all the genes tested in each cell line are expressed at relative high abundance compared to β -actin, a housekeep gene that are expressed at a very high level (Figure 27B). At the protein level, STAT1, a major transcription factor that mediates the effects of type I IFNs, was readily detected with its antibody in 10T1/2 cells and D3 cells, with a slightly higher expression level in D3 cells as determined by flow cytometry (Figure 27C).

Different Induction Patterns of ISGs in mESCs and 10T1/2 Cells

To further characterize ISG expression, their induction patterns in IFN β treated mESCs and 10T1/2 cells in a 24 h time course were compared. As shown by Figure 28,

the mRNA of *Pkr* and *Isg15* were quickly induced in both cells by IFN β at 4 h. However, they quickly declined to ~50% of the maximal activation in 10T1/2 cells at 8 h, but the mRNA level of the two genes in mESCs were sustained (Figure 28, A and B).

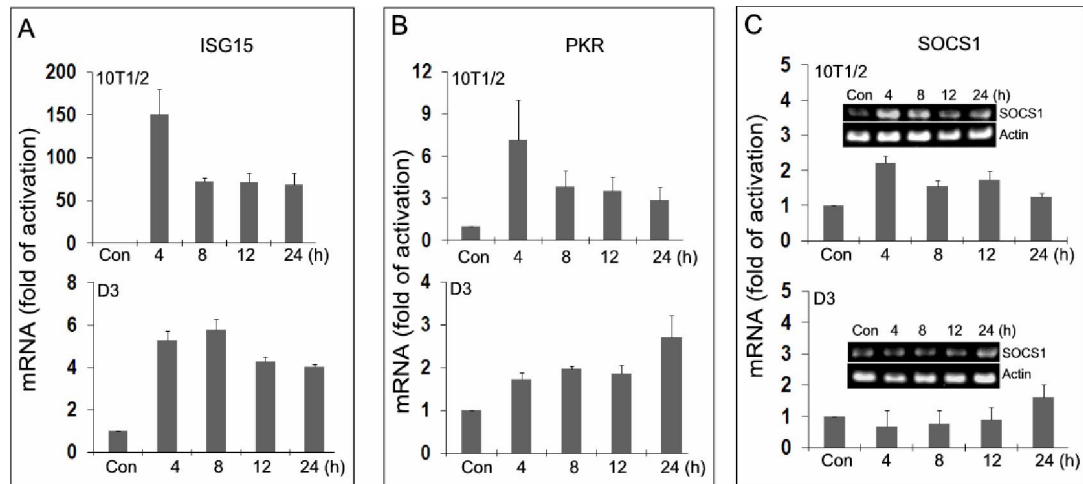


Figure 28. Expression patterns of ISGs in mESCs and fibroblasts. D3 and 10T 1/2 cells were treated with 5000U/ml IFN β for indicated time points as indicated. The relative mRNA levels of ISG15 (A), PKR (B), and of SOCS1(C) were analyzed by RT-qPCR. The results are expressed as fold-activation where the mRNA level in control cells is designated as 1. The values in A and C are means \pm SD of three independent experiments. The values in B and C are means \pm SD of three independent experiments. The values of A are means \pm SD of a representative experiment performed in triplicates. The experiments were performed twice with similar results. The gel insets in C are RT-PCR products of SOCS1 analyzed by agarose gel electrophoresis. The control (Con) represents cells without IFN β treatment.

The author further tested the expression patterns of *Socs1*, a negative regulator of IFN signaling. As shown in Figure 28C, *Socs1* was induced by IFN β at 4 h in 10T1/2 cells, but such induction was not observed until 24 h in mESCs. It is well established that SOCS1 strongly inhibits ISG expression, thereby limiting the strength and duration of IFN response (Kubo, Hanada, & Yoshimura, 2003). The expression pattern of *Socs1* explains the induction dynamics of *Pkr* and *Isg15* in 10T1/2 cells since the induction of *Socs1* at 4 h coincided with the decline of *Pkr* and *Isg15*. On the other hand, delayed

induction of *Socs1* in mESCs could account for the sustained expression of *Pkr* and *Isg15* (Figure 28, A and B). While IFN β induced ISGs and SOCS1 in 10T1/2 cells displayed a typical patterns as seen in other differentiated cells, the results from mESCs are “unconventional” although the basal mRNA level of *Socs1* is comparable in two cell types.

IFN β Potentiates dsRNA-inhibited Proliferation of mESCs

PolyIC has been shown to activate PKR, thereby inhibiting mESC proliferation, although it cannot induce IFN β in mESCs (Wang et al., 2013).

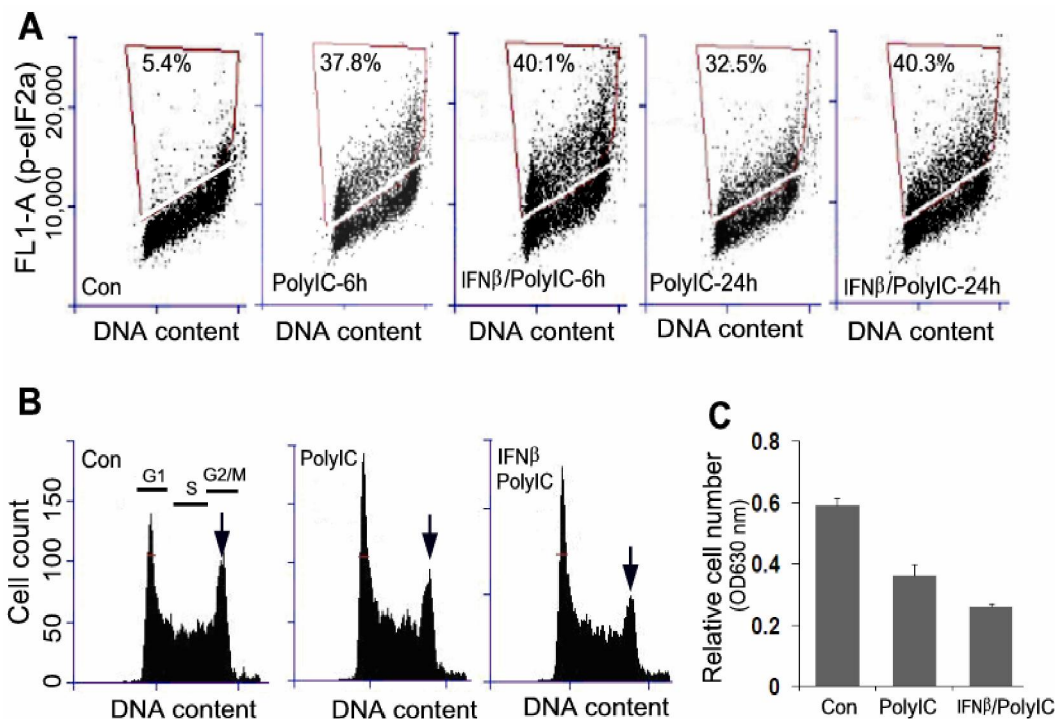


Figure 29. IFN β potentiates polyIC-inhibited proliferation of mESC. **A**, D3 cells were transfected with 300 ng/ml polyIC alone (PolyIC), or pretreated with IFN β 5000 U/ml) for 24 h followed by polyIC transfection (IFN β /PolyIC). The cells with phosphorylated eIF2 α (p-eIF2 α) were quantified by flow cytometry (boxed areas. White slash lines were used to help identify the bottom sides of the boxes) at the times indicated. **B**, The cells treated under the condition described in **A** at 24 h were analyzed for cell cycle by flow cytometry. The change in G2/M phase cells was indicated by the arrow. **C**, The numbers of viable cells in the samples described in **B** were determined by toluidine blue cell

staining. The values are means \pm SD of a representative experiment performed in triplicates. The experiments were performed at least twice with similar results. The control (Con) represents cells without any treatment.

Since IFN β induces PKR expression, the author assumed that treatment of mESCs with IFN β would augment the effect of polyIC. To test this hypothesis, PKR activity was determined by the phosphorylation of eukaryotic initiation factor 2 α (eIF2 α , a known substrate of PKR) (Wang et al., 2013).

As shown in Figure 29, polyIC transfection caused eIF2 α phosphorylation in mESCs at 6 h and 24 h, which were potentiated in the cells that were pretreated with IFN β (Figure 29A, PolyIC and IFN β /PolyIC, respectively, boxed areas). Accordingly, polyIC by itself caused a significant reduction of cells in the G2/M phases, which was further augmented by IFN β (Figure 29B, PolyIC and IFN β /PolyIC, respectively, indicated by arrows). The inhibitory effect on cell cycle was reflected by reduced cell proliferation where IFN β pretreatment caused slight but statistically significant additional effect to polyIC alone (Figure 29C). These results further confirm the functionality of the molecular mechanisms that mediate the effects of IFN β in mESCs.

Type I IFNs Do not Affect Unique Properties of mESCs

Type I IFNs have been best characterized as immunomodulators, but they also regulate other cellular processes (Platanias, 2005). To test if they have any effect on the properties of mESCs, the effects of IFN β and IFN ω on the expression of pluripotency markers and cell proliferation were examined. mESCs are characterized by their rapid cell proliferation rate with about 60% of cells in the S phase, and they grow in compacted colonies (Stead et al., 2002).

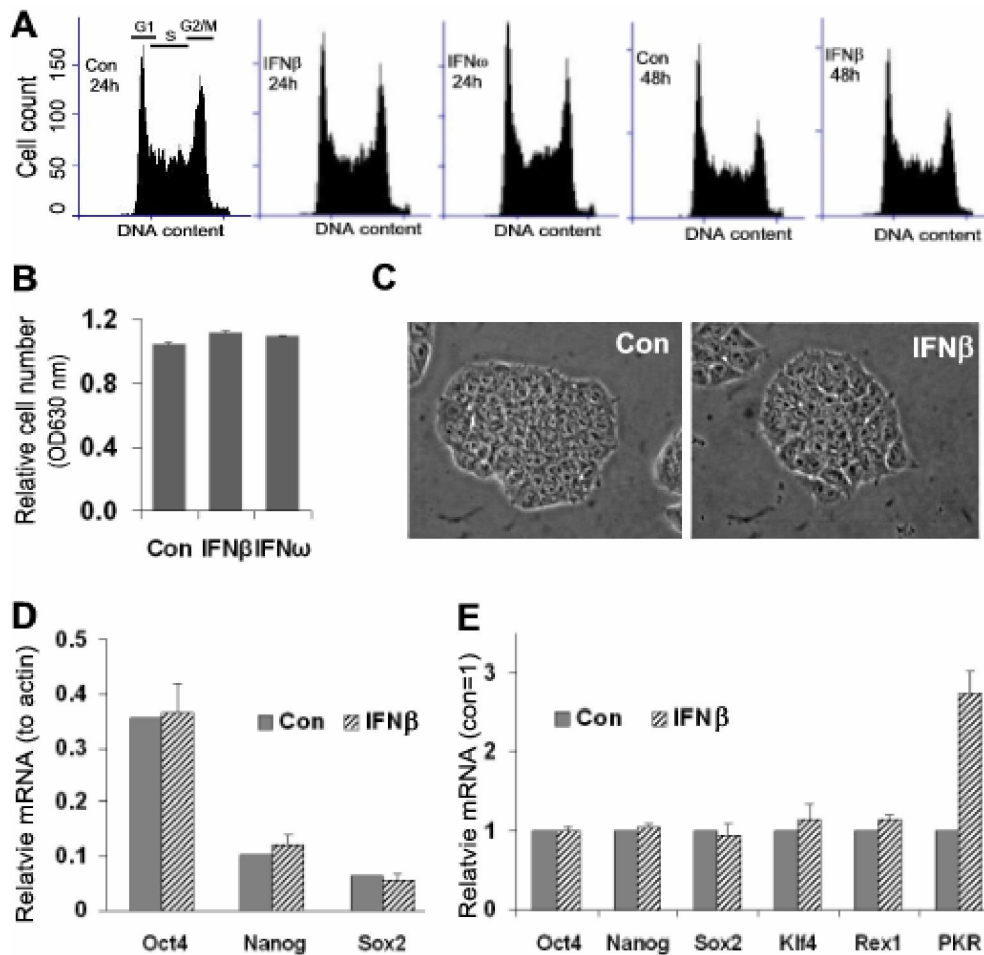


Figure 30. Type I IFNs do not affect the stem cell state of mESCs. D3 cells were treated with IFN β or IFN ω (5000 U/ml) for 24 h or 48 h. The cells were then analyzed for: A, cell cycle progression by flow cytometry, B, cell proliferation by toluidine blue cell staining (24 h treatment), C, cell/colony morphology analysis by microscopy (48 h treatment), D and E, the mRNA levels of pluripotency markers by RT-qPCR in the cells that were treated with IFN β for 48 h once (D) or twice (2x 48h) (E). The results are means \pm SD of three independent experiments (D) or a representative experiment performed in triplicates (E). PKR was used as a positive control. The control (Con) represents cells without IFN β treatment.

As shown in Figure 30, neither IFN β nor IFN ω affected the cell cycle profile (A), cell proliferation (B), or colony morphology (C). Similarly, the expression levels of the pluripotency markers unique to ESCs were not affected either by a short period single treatment with IFN β (Figure 30D, 48 h) or by two consecutive treatments, while PKR (an ISG as a positive control) was induced by IFN β treatment (Figure 30E, 2x 48h). Similar

results were obtained in DBA cells (data not shown) or when the cells were treated with IFN β . Therefore, type I IFNs do not affect the stem cell state of mESCs.

Discussion

The ability to produce and respond to type I IFNs as a critical component of innate immunity is presumably developed in all types of mammalian cells. However, our studies in mESCs and those of other investigators in hESCs have revealed that these cells are deficient in expressing type I IFNs, indicating that the “innate immunity” is not, or at least not fully, developed in ESCs (Chen et al., 2010; Wang et al., 2013). This finding revealed a unique property of ESCs that has not been previously characterized. In this chapter, the responses of mESCs to type I IFNs were investigated.

A recent study reported that hESCs have substantially attenuated response to IFN β as judged by their negligible expression of ISGs, but the antiviral activity of IFN β was not tested against live viral infection (Hong & Carmichael, 2013). However, it is interesting to note that two early brief studies in mESCs have indicated that these cells can respond to IFN β , but they attracted little attention in the early days of ESC research (Ruffner, Reis, Naf, & Weissmann, 1993; Whyatt, Duwel, Smith, & Rathjen, 1993). The molecular mechanisms involved and physiological implications of these findings in ESCs are not known. Based on multiple criteria, the results presented in this chapter demonstrate that mESCs have the basic functional mechanisms to respond and to mediate the effects of type I IFNs. At the cellular level, IFN β and IFN ω can protect the cells from LACV-induced lytic cell death and repress viral replication of LACV and

WNV. At the molecular level, mESCs express the major signaling components of the IFN pathway. More importantly, mESCs are able to express ISGs, which is the hallmark of IFN action and the molecular basis for the antiviral activity of type I IFNs.

Therefore, IFN β and IFN ω can induce major responses in mESCs like in differentiated cells although the magnitude of ISG induction in mESCs is generally lower than in fibroblasts.

In hESCs, the major signaling molecules of the IFN pathway are expressed at relatively lower levels than in differentiated cells; however, the failure of hESCs to respond to IFN β is mainly attributed to the high expression level of suppressor of cytokine signaling 1 (SOCS1) (Hong & Carmichael, 2013). In differentiated cells, SOCS1 is expressed at a low basal level in the resting cells, but it is rapidly induced and acts as a negative regulator of JAK/STAT signaling upon stimulation with IFNs or viral infection (Kubo et al., 2003). However, hESCs constitutively express high levels, thereby repressing IFN β action (Hong & Carmichael, 2013). Transcriptional analysis in mESCs revealed that, with the exception of *Ifnar2*, the mRNA of the major signaling molecules in the IFN pathway are expressed at comparable or even higher levels than in fibroblasts (10T/12 cells), whereas the mRNA of *Socs1* is expressed at a basal level in mESCs that is similar to 10T/12 cells. These results could explain the responsiveness of mESCs to type I IFNs and the difference between mESCs and hESCs.

Currently the reasons for the low levels of ISG induction by IFN in mESCs are still elusive, but this result is not surprising due to the multiple level regulation of ISG

expression. It is likely that some mechanisms required for maximal ISG expression may have not yet fully developed in mESCs. It is also possible that IFN response systems in mESCs and differentiated cells could be modulated via different regulatory mechanisms. However, a more plausible reason could be closely related to the defective IFN expressing mechanism in mESCs. In differentiated cells, the cellular responses induced by exogenous IFN β can be strongly potentiated by the cellular expressed IFNs through autocrine signaling (Samuel, 2001; Stetson & Medzhitov, 2006). Therefore, in IFN primed (or virally infected) 10T1/2 cells, the ISG induction is the collective effects of exogenously added IFN β and endogenously expressed cellular IFNs, whereas the ISG induction in mESCs is solely due to exogenously added IFN β since these cells are deficient in expressing type I IFNs (Wang et al., 2013).

In differentiated cells, it is known that the signaling molecules in immune responses are upregulated by the initial stimulus via a positive feedback loop (Huang et al., 2006; Matsumoto & Seya, 2008; Pan et al., 2011). Through paracrine and autocrine signaling mechanisms, IFN β induced from the initial antiviral response can boost the antiviral responses of the infected cells and prime uninfected cells to enter the antiviral state (Samuel, 2001; Stetson & Medzhitov, 2006). A critical part of this mechanism is the upregulation of viral sensing receptors in primed cells. Using polyIC as viral stimuli, the upregulation of *Rig-I* and *Tlr3* was detected in IFN β primed mESCs, thereby demonstrating the existence of priming mechanism in these cells. Based on these observations, it can be concluded that the priming system in mESCs is operational. It is

noted that priming does not result in an increase of cellular IFN β expression in response to polyIC, suggesting that deficiency of IFN β expression cannot be corrected or compensated for by priming with exogenously added IFN β . Therefore, such deficiency is likely a developmental issue related to the stem cell state.

Pretreatment of mESCs with IFN β confers strong protection against LACV infection, as judged by the reduced cytotoxicity and viral replication. However, the antiviral effect of IFN β in inhibiting the replication of WNV in mESCs is much lower than in 10T1/2 cells. The reasons for this discrepancy are not clear but could be explained by several possibilities. For example, it is known that the antiviral activities of IFN β are cell type as well as virus dependent (Goodbourn, Didcock, & Randall, 2000; Li & Sherry, 2010; Rose & Weiss, 2009). As previously reported, although the IFN expressing system is deficient, PKR can be activated by LACV infection, which could contribute to the antiviral activities of IFN β in LACV-infected mESCs (Wang et al., 2013), whereas, WNV infection did not activate PKR in mESCs as in other rodent cells (Elbahesh et al., 2011; Wang et al., 2013). It is possible that the lack of PKR activation in WNV-infected mESCs may account for the weaker antiviral activity of IFN β .

In addition to participating immune responses, type I IFNs also regulate several other important biological processes (Platanias, 2005). Whether and how they affect the unique properties of ESCs have not been previously investigated. The results suggested that neither IFN β nor IFN ω treatment affected the three distinctive features of mESCs – rapid cell proliferation rate, colony morphology, and pluripotency. However, it should be

pointed out that these experiments were conducted in the presence of LIF that represses cell differentiation. The outcome could be different if the experiments are conducted with differentiating cells in the absence of LIF.

hESCs and mESCs share fundamental similarities in pluripotency and self renewal, but they also show species differences in two important aspects. First, activation of the JAK/STAT3 pathway by LIF (which shares the similar signaling paradigm with IFN) is essential for the maintenance of self-renewal and pluripotency in mESCs, but is not required for hESCs (Daheron et al., 2004; Humphrey et al., 2004; Matsuda et al., 1999). Second, mESCs are characterized by a shortened cell cycle whereas hESCs have time frame similar to differentiated cells (Burdon, Smith, & Savatier, 2002; Dalton, 2009; Singh & Dalton, 2009). The difference in responding to type I IFNs in mESCs (this study) and in hESCs represents a new distinctive feature in ESCs from the two species (Hong & Carmichael, 2013). At the present time, the implications of this difference in embryo development and reasons for this discrepancy in human and mouse remain to be determined.

In summary, this study demonstrates that cellular mechanisms in type I IFN production and action are differentially developed in mESCs (Wang et al., 2013). They are deficient in expressing type I IFNs but have the basic mechanisms to mediate the cellular effects of type I IFNs although these mechanisms may not yet be fully developed. These findings imply that, while mESCs are unable to produce type I IFNs to confer antiviral activities to themselves and their neighboring cells, they are able to acquire

antiviral effects from type I IFNs secreted from other cells (via paracrine mechanism).

While it is not clear about physiological implications for mESCs not having the ability to produce type I IFNs, being able to acquire the antiviral activities from these cytokines could be an advantage for mESCs. These findings bring up an important subject in ESC biology for understanding the development of innate immunity during embryogenesis.

CHAPTER VI

CONCLUSION

In the three related projects, the author first studied the immune responses of mESCs to live viruses and different synthetic viral RNA analogues, with emphasis on their ability to express type I IFN. Then examined how mESCs respond to type I IFN and tested whether they can be protected by IFNs from viral infection were examined. Overall, the findings confirmed deficient anti-viral response which is centered by induction of type I IFN in mESCs although they are partially responsive to type I IFN and the following protection from certain types of viral infection. Together with the findings in hESCs and in other pluripotent cells in early years, it can be concluded that the deficiency in type I IFN signaling is likely a characteristic of mammalian pluripotent cells (Burke, Graham, & Lehman, 1978; Chen et al., 2010; Hong & Carmichael, 2013). While the type I IFN-related anti-viral activity is under-developed in pluripotent cells, they have been reported to utilize RNA interference (RNAi) to combat viral infection (Li, Lu, Han, Fan, & Ding, 2013; Maillard et al., 2013). During natural development, differentiated somatic cells acquire fully functional type I IFN signaling which becomes the major anti-viral player of innate immunity, while anti-viral effects of RNAi diminish, as supported by the finding that defective RNAi mutation in differentiated cells does not rescue the growth of viruses with mutant suppressor of RNAi (Pare & Sullivan, 2014). In human differentiated cells, Argonaute2 (Ago2) which is a key component of RNA induced suppressor complex (RISC), is inhibited during stress and pathogen infection (Leung et al., 2011; Mazumder,

Bose, Chakraborty, Chakrabarti, & Bhattacharyya, 2013; Seo et al., 2013). Pluripotent stem cells seem to use different strategies from their progeny to fight against viral infection.

It is interesting to note that IFN pathways are evolutionarily young and only exist in vertebrates, while RNAi is widely used by many metazoans including plants and invertebrates to combat viruses. Thus, mammalian pluripotent cells seem to share anti-viral strategies with invertebrates and plants. The advantage of RNAi anti-viral strategy in pluripotent cells may lie in avoiding side effects of IFN signaling on proliferation, higher efficiency in defending against transposons and preventing sacrifice of the lineage due to some harmless triggers such as cytoplasmic cellular dsRNA, which can be detected in pluripotent cells (Chen & Carmichael, 2009; Chen et al., 2010; Hertzog, Hwang, & Kola, 1994).

The current research just begins to reveal the anti-viral responses in pluripotent stem cells. More importantly, the development of anti-viral immunity and fully functional type I IFN system is still elusive and requires a tremendous amount of further exploration.

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